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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) This report describes the effects of treatment with low levels of the cholinesterase (ChE) inhibitors Sarin (0.5 LD50 s.c. 3 times weekly) and pyridostigmine bromide (PB, 80 mg/L in drinking water) alone or in combination for 3 weeks as compared with untreated controls. At 2, 4 and 16 weeks after exposure, the following tests were performed: (1) Conditioned avoidance response (CAR), a test of learning and memory; (2) measurements of arterial blood pressure, heart rate, and baroreceptor reflexes; (3) measurements of regional cerebral blood flow (rCBF). There was a significant decrease in whole blood and RBC cholinesterase activities during treatment that returned to normal between 2 to 3 weeks after treatment. CAR, arterial blood pressure, heart rate, and measurements of baroreceptor mechanism gain did not show significant differences between experimental groups. rCBF indicated significant enhancement of cerebral perfusion in neocortex (face, hindlimb, forelimb, and trunk areas, primary and secondary motor areas, auditory and visual cortex), with a few additional locations with significant elevation of rCBF in entorhinal, ectorhinal, and piriform cortex in animals treated with Sarin+PB 2 weeks after treatment. At 4 weeks after treatment, the same general pattern was found in animals treated with sarin, with more significant locations in piriform and retrosplenial cortex, as well as amygdala. Only few changes in rCBF were found at 16 weeks post-treatment in the three experimental groups. In conclusion, learning, memory and cardiovascular regulations of the animals under study were not altered by the treatments used. Changes in rCBF were observed for sarin, alone or in combination with PB, but did not persist beyond the fourth week after treatment.				
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INTRODUCTION.

Organophosphorus (OP) cholinesterase (ChE) inhibitors are among chemical weapons to which army personnel and civilians could be exposed, at symptomatic or sub-symptomatic doses. The carbamate ChE inhibitor pyridostigmine bromide (PB) has been fielded as a prophylactic treatment against OP ChE inhibitors by the US Armed Forces and used in the Persian Gulf War. Although acute intoxication with OP ChE inhibitors and the protective effect of PB on this phenomenon have been extensively studied in animals, the potential long term harmful effects of low level (subsymptomatic) exposure to OP ChE inhibitors, alone or in combination with PB have received little attention. This is the objective that the present proposal intends to address.

In our experimental approach to this objective, we are evaluating the possible occurrence of delayed neurologic dysfunction after exposure of animals to PB or to doses of the OP cholinesterase inhibitor sarin, low enough to be free of acute toxic effects, alone or in combination with PB treatment. During the previous year of support, inhibited (passive) avoidance and open field activity were used to assess cognitive function, motor activity, and habituation. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation respectively, as well as the expression of muscarinic cholinergic receptors. These assays were performed in the same animals that were subjected to the neurobehavioral tests mentioned above.

These studies were preceded by experiments aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs following single or multiple doses within the three week period of treatment. In the case of PB, the optimal dose was defined as one producing 20-30% inhibition of plasma butyrylcholinesterase (BuChE). This is the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf war ¹ (90 mg PB over 24 hrs, divided in three oral doses).

During the second year of support, we continued the study of cognitive function after exposure to subtoxic doses of cholinesterase inhibitors with the same experimental design described above, using the conditioned avoidance test². In addition, the possible existence of neurologic dysfunction in the exposed animals was tested by a study of the baroreceptor reflex, a well characterized autonomic nervous system regulatory mechanism that includes peripheral as well as central cholinergic mechanisms ^{3,4}. The effects of pharmacological challenges that increased or decreased arterial blood pressure acutely was quantified to characterize the gain of the baroreceptor reflex and the incidence of heart arrhythmias. Finally, regional cerebral blood flow (rCBF) was measured with the Iodo-¹⁴C- antipyrine technique in order to produce cerebral functional activation maps.

MATERIALS AND METHODS.

1. Animals.

Male Crl:CDBR Vaf/Plus Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity ($50 \pm 10\%$) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and water were freely available. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2. Materials.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical and Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously. PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water at a concentration of 80 mg/L and provided as drinking water to experimental groups for a three-week period.

3. Experimental Procedures.

Animals were exposed to treatments (saline, sarin, PB or sarin+PB) during three weeks at the US Army Institute of Chemical Defense laboratory in Aberdeen Proving Ground (APG). After a period of 1 to 15 weeks following treatment, depending on the experimental groups, they were transported by air-conditioned vans and air-freight to the Veterans Affairs Greater Los Angeles Healthcare System (VA GLAHS) laboratory where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables.

Blood cholinesterase measurements.

When animals were received at the ICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein by the method described by Liu et al.⁵ on two separate days to establish baseline whole blood and red blood cell (RBC) cholinesterase (ChE) activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday at 10:00AM (about 60 min after sarin or saline injections) during the 3-week exposure period and continued for 3 more weeks during the recovery period.

Blood was collected into an Eppendorf 1.5 ml microtube containing 50 μ l (1000 USP unit per ml) heparin sodium and mixed. Forty μ l of whole blood was transferred to another microtube containing 160 μ l 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off and 20 μ l RBC's was

transferred into a microtube containing 180 μ l 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at -75°C until ChE analysis.

Whole blood and RBC ChE activity were determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Inc., Nutley, NJ). The analytical procedure was based on the manual method of Ellman⁶ and modified for the COBAS/FARA system using acetylthiocholine as substrate.

Anesthesia for surgical procedures: Animals were anesthetized by exposure to 2.5% halothane in air in a closed plexi-glass chamber with continuous flow of gas from an anesthesia machine. After 2-3 minutes the animal was transferred to a table provided with a heating pad, and a maintenance concentration of Halothane (1.5%) was given by mask throughout the surgical procedure. A scavenging system (Fluosorb) prevented excess halothane from reaching the environment. The concentration was raised if withdrawal to painful stimulation was observed. Anesthesia was discontinued after surgical wounds were sutured. The condition of the animal was monitored frequently during the post-operative period.

Measurement of arterial blood pressure, and heart rate at baseline and in response to drug interventions: Femoral arteries and veins were cannulated with PE50 polyurethane (artery) and 0.64 mm O.D. silastic (vein) catheters under halothane anesthesia, and the animals were allowed to recover in a Bollman cage, after

discontinuation of the anesthetic, for 45 min. Arterial blood pressure (ABP) was recorded with a pressure transducer interfaced to a Hewlett-Packard polygraph. The output from this instrument was digitized and saved with a data acquisition system (Axotape, Axon, Inc.) for off-line analysis with custom-written Matlab scripts (MATLAB, Inc). Arterial blood pressure (ABP) was transiently altered by pulse injection of phenylephrine (5 to 10 $\mu\text{g/kg}$, i.v.) and sodium nitroprusside (20 to 50 $\mu\text{g/kg}$, i.v.). Heart rate (HR) was extracted off-line from ABP records, and regressions of HR on ABP were calculated from data obtained before and after the pulse injections of phenylephrine and nitroprusside, as an estimate of the baroreceptor gain.

Measurement of cerebral blood flow: Regional cerebral blood flow (rCBF) was measured with the Iodo- ^{14}C -antipyrine (^{14}C -IAP) quantitative autoradiographic method ⁷. Two arterial and two venous catheters were implanted in the femoral vessels under halothane anesthesia used as described above. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for one hour. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp, Inc.) temperature controller and a source of radiant heat. One arterial catheter was connected to a pressure transducer interfaced to a polygraph for continuous recording of arterial blood pressure, the other one was used for sampling of arterial blood. One of the venous catheters was

connected to a motor driven syringe containing the radioactive tracer solution and the other one to a similar syringe containing the euthanasia solution (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus). A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then the infusion of ^{14}C -IAP was started. Infusate volume was 0.6 ml, dose 100 $\mu\text{Ci/kg}$ and infusion period 30 seconds. Arterial blood samples (30 μL) were obtained every three seconds from a free flowing catheter. Circulation was arrested by the euthanasia solution delivered intravenously over the last 4 seconds of the ^{14}C -IAP infusion. The exact timing of circulatory arrest was determined from the polygraph record of arterial blood pressure. The brain was then rapidly removed and flash frozen in methyl-butane chilled to -70°C . These tissues were sectioned in a cryostat at -20°C in 20 μm slices, heat-dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with 8 standards of known radioactivity to obtain an ^{14}C -IAP autoradiogram. rCBF was calculated from film optical density of brain autoradiographs and standards, and arterial blood radioactivity as described previously ⁷

Conditioned avoidance response: A discrete trial, one-way conditioned avoidance response was observed using a previously described procedure ². Two responses were studied: an innate escape response and a learned avoidance response. There was a maximum of 30 trials per session, with two sessions 24 hrs apart. The number of animals reaching criterion (6 consecutive avoidance responses) and the average escape and avoidance times per animal in both sessions were recorded for all experimental groups.

4. Experimental Design.

4.1. Experimental groups.

Separate sets of animals were studied 2, 4, and 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Number of animals was 12 per treatment group, as determined by statistical power analysis, and the total number of groups (treatments x times after treatments) 12, with a grand total of 144 rats.

Treatment group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline. Treatment group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. Treatment group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5 LD₅₀). Treatment group 4 animals received PB in drinking water and were injected with sarin. PB in drinking water was provided continuously to groups 2 and 4 animals starting on Monday morning at 0800 hour. At 0900 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of 6 animals per dose. PB in drinking was terminated and switched to regular tap water at 1700 hour on Friday of the third week. Animal dosing procedures were performed at the APG laboratory. All animals were then shipped to the VA GLA laboratory location, where the planned main biochemical and behavioral studies in these animals were performed 2 weeks, 1 month, and 4 months after sarin, sarin + PB, PB, or control treatments.

4.2. Data Analysis.

Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data is presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each interval after exposure to drugs or saline with one factor (treatment) at four levels (saline, PB, sarin, sarin+PB). This analysis was followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

KEY RESEARCH ACCOMPLISHMENTS (RESULTS).

Dose Finding Studies: Experiments carried out at the GLA VA during the first year of the study indicated that animals drinking water with PB at a concentration of 80 mg/L had inhibition of plasma butyrylcholinesterase slightly below 80% of baseline on average. This was within the target effect set for these experiments (20 to 30% inhibition). The next higher PB concentration in drinking water (160 mg/L) induced a larger BuChE inhibition (between 59 and 75% of baseline) and inhibition of RBC ChE between 49 and 57% of baseline. Thus the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No signs of toxicity, as defined in Section 4, were found in animals treated with PB.

The dose finding for sarin, and the combination of sarin and PB carried out at Dr Shih's laboratory in Aberdeen Proving Grounds (APG) during the first year of the study

indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects when given alone or in combination with PB (80 mg/L in drinking water).

Body mass: Means of body mass, recorded daily during weekdays, through the three weeks of treatment and the following two weeks at the APG laboratory showed no statistically significant differences between treatments. The means and SE of body mass at the beginning of the experiments that assessed outcome variables, when the animals were already at the GLA VA laboratory, showed the expected increase in body mass with age, but no differences among treatment groups (Table 1).

Blood cholinesterase activity: Measurements of RBC and whole blood ChE during drug treatment (weeks 1, 2 and 3) and the immediate recovery period (weeks 4, and 5) are shown in Figs 1 and 2, respectively. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following two weeks of treatment, and completely after treatment ceased. Sarin produced a decrease in RBC and whole blood ChE activity to about 40% of baseline that remained stable during the treatment period, and recovered to values not statistically different from the control group by the end of the second week after drug treatment ceased. The association of PB and sarin induced a greater depression of RBC ChE activity that persisted until the second week after treatment (Fig 1).

Arterial blood pressure, blood gases, and body temperature: ABP was recorded under two conditions: a) prior to measurements of baroreceptor responses, and b) prior to

measurement of cerebral blood flow (see below). The results of the first set of measurements are shown in Fig 3, and those of the second set, in Table 1. No significant differences between means of the four treatment groups were found in any case.

Cardiovascular regulation: Typical responses of BP and HR to phenylephrine and nitroprusside are shown in Fig 4. The highest phenylephrine dose elicited atrioventricular blockade (Fig 4, top) followed by nodal, and in some cases ventricular ectopic rhythms. The coefficient of the regression of HR on BP, calculated from hypertension data prior to the A-V block, yielded values similar to that of the regression obtained from hypotensive episodes. For that reason both sets of data were pooled in one analysis (Fig 5). In another analysis, only data from hypertensive episodes, including the period of A-V block, was used (Fig 6). None of the differences between experimental groups reached statistical significance.

Cerebral blood flow: Regional cerebral blood flow (rCBF) was measured with the Iodo-¹⁴C-antipyrine technique (see methods). The following regions, identified according to the Atlas of Paxinos and Watson, were sampled: amygdala (Am), auditory cortex (Au), primary auditory cortex (Au1), barrel cortical field (BF), ectorhinal cortex (Ect), entorhinal cortex (Ent), face cortical area (Fa), forelimb cortical area (FL), hindlimb cortical area (HL), insular cortex (I), primary motor cortex (M1), secondary motor cortex (M2), parietal association area (PA), piriform cortex (Pir), retrosplenial cortex (RS), primary somatosensory cortex (S1), secondary somatosensory cortex (S2), temporal cortex (Te), trunk cortical area (Tr), primary visual cortex (V1), and secondary visual cortex (V2). Means of CBF of every region are displayed in Figs 8-10 in three

dimensional maps in which the ordinate represents distance along the rostro-caudal axis of the brain, the abscissa position of regions relative to the midline, and mean CBF of every cell is represented on a color scale. Statistical significance against the control group is indicated in these graphs by white ovals ($P < 0.05$, Bonferroni adjusted for three contrasts). At 2 weeks after treatment, significant changes were only observed in animals treated with sarin+pyridostigmine. The regions affected were located mostly on the neocortex (Fa, M2, S2, BF, FL, HL, Te, Au, Au1, V1, V2), with a few on Ent and Ect and only one on Pir. At 4 weeks after treatment, the same general pattern was found in animals treated with sarin, with more significant locations in Pir, RS, and Am. Only few changes were found at 16 weeks post-treatment in the three experimental groups.

Arterial blood gases, pH, and arterial blood pressure, measured at the time of rCBF measurements, did not show any significant differences with regard to controls for any of the experimental groups. Thus differences in rCBF reported could not be explained by changes in these variables. Body temperature was slightly higher in all PB group means, and it reached statistical significance at 2 and 4 weeks post-treatment (Table 1). The expected variation of body mass with age was found at this time, but no differences among groups were detected within a given time after treatment.

Conditioned avoidance: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test and the same parameters for animals that gained or lost criterion in the second day with regard to the first are shown in Fig. 7, top and bottom panels, respectively. No

significant difference was detected among experimental groups for the pooled data shown in Fig. 7, nor for any of the time points after treatment.

DISCUSSION AND CONCLUSIONS.

The experiments reported here have addressed the delayed effects of repeated low dose administration of cholinesterase inhibitors on three fundamental functions of the cholinergic system in the central nervous system: (1) cognition, (2) autonomic regulation of the cardiovascular system, and (3) control of the cerebral circulation. The approach has been to measure (1) with the conditioned avoidance response, which complements information gathered previously in this project with the passive avoidance response, and open field activity, (2) with the baroreceptor reflex and recordings of basal heart rate and arterial blood pressure, and (3) with mapping of cerebral cortical blood flow with the quantitative autoradiographic Iodo-¹⁴C-antipyrine technique. Because the cerebral cholinergic system, one of several neurotransmitters system involved in the functions mentioned above, is the main target of cholinesterase inhibitors, we tested the hypothesis that those functions would show deviations from normality in animals treated with low-level ChE inhibitors.

The acute effects of low-dose ChE inhibitors on behavioral parameters reported in the literature are contradictory. No effect of soman on learning and memory was found by Russell et al. ⁸, who used the same methodology (conditioned avoidance) as in the present report, while Kassa et al. have reported a deficit in Y-maze performance that subsided three weeks after exposure to low-dose sarin ⁹. The results reported here indicate a lack of effect of the treatment regimes used on the conditioned avoidance

response. This is in line with results reported for the first year of this program that indicated lack of effects of the same treatments on passive avoidance behavior. Evidently even if any cognitive alterations occurred with low level sarin or pyridostigmine treatments, they appear to have subsided by the time of the first tests at 2 weeks.

Regarding cardiovascular regulation, Administration of low-dose ChE inhibitors induces hypertension, a phenomenon first shown for physostigmine in rats ¹⁰. The same is true for high dose soman ¹¹. This phenomenon is mediated by central activation of muscarinic receptors, leading to enhanced adrenergic output ¹². The pressor response described above is usually preceded by bradycardia, that can be prevented by peripheral muscarinic blockade ¹³.

The baroreceptor reflex has been tested in animals given the carbamate ChE inhibitor physostigmine by several laboratories. The hypertensive response that follows bilateral carotid occlusion in rats, due to unloading of the carotid sinuses, is potentiated by systemic, as well as central administration of physostigmine ^{14,15}. The reflex bradycardia resulting from transient hypertension induced by nor-epinephrine is enhanced by physostigmine, while the reflex tachycardia resulting from transient hypotension induced by nitroprusside is reduced ¹⁶. The response to head-up tilt consists of a rapid decrease in arterial blood pressure, followed by a slower decline, with a transient increase on resuming the horizontal position. Central administration of physostigmine attenuated the tilt-induced decreases in MAP, but it did not affect the restoration-related transient pressor response ¹⁵. No information is available in the

literature regarding effects of OP ChE inhibitors on these regulatory responses. In the present series of experiments, no alterations have been found on the resting levels of arterial blood pressure and heart rate, and no changes have been detected in the gain of the baroreceptor mechanism.

The changes observed in the regional distribution of cerebral blood flow were of a small magnitude (10 to 20% of control in selected regions) when compared to the acute effects of OP¹⁷ or tertiary carbamate^{18,19 20,21}, ChE inhibitors (up to 200% of controls). The only significant changes at 2 weeks after treatment were found in the group treated with a combination of sarin and pyridostigmine bromide. It is possible that some remaining ChE inhibition may have persisted in compartments associated with blood vessels at this time, since a significantly lowered RBC ChE was observed at 2 weeks after treatment in the same group (Fig 1). However, the enhanced rCBF observed in neocortex and some areas of allocortex, 4 weeks after treatment, it is not easy to explain, in particular because such change was not observed at the earlier 2 week period. It is tempting to speculate that this increase may have been related to enhanced arousal, known to be associated with enhanced rCBF, as a rebound phenomenon following the depression related to acute effects of sarin. Indeed our previous observations have indicated a decrease in mobility of sarin treated animals at 2 weeks after treatments that subsided at the 4 week testing period. Elucidation of this possibility will have to await the conclusion of experiments, being carried out during the current year, in which we are measuring the regional levels of cerebral glucose utilization in the same regions used for rCBF measurements, and under similar experimental regimes. The rCGU methodology

evaluates the level of neuronal activation, while rCBF changes reflect, in addition to neuronal activation, any direct influences that may exist on cerebral vascular smooth muscle. A pattern of rCGU similar to the observed pattern of rCBF changes will argue for an effect on neuronal activation of the regions involved, while lack of rCGU enhancement in the areas in question will tend to indicate a direct vascular effect, possibly related to persistent ChE inhibition. It is noteworthy that no consistent changes in rCBF distribution were observed beyond 4 weeks after treatment.

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Treat.	Weeks	Blood pH -log[H ⁺]	Pa CO ₂ (mmHg)	PaO ₂ (mmHg)	Body mass (g)	Body temp (°C)	MABP (mm Hg)
Control	2	7.447±0.002	40.4±0.8	88.5±1.5	440.3±10.3	37.5±0.2	118.0±4.1
PB	2	7.440±0.003	39.4±0.8	87.4±1.9	449.2±14.2	38.2±0.2	110.2±3.4
Sarin	2	7.439±0.008	39.7±0.9	84.8±2.7	475.0±8.6	37.6±0.1	122.5±2.8
Sarin+PB	2	7.448±0.011	40.0±1.3	89.2±1.7	456.2±7.6	37.9±0.2	120.4±3.2
Control	4	7.450±0.004	39.6±0.9	88.7±1.0	461.3±11.5	37.5±0.2	119.5±2.4
PB	4	7.452±0.007	39.8±0.8	85.8±1.0	498.2±12.8	38.3±0.3	121.4±6.4
Sarin	4	7.442±0.005	38.4±0.7	85.5±1.5	469.1±9.2	37.9±0.3	117.5±3.8
Sarin+PB	4	7.441±0.005	40.2±0.7	92.4±1.5	483.3±14.6	37.7±0.1	126.2±3.3
Control	16	7.440±0.007	39.2±0.6	89.3±0.9	596.5±13.1	37.6±0.2	110.6±4.0
PB	16	7.428±0.008	39.0±0.4	85.4±1.6	582.8±24.6	38.0±0.5	120.4±3.5
Sarin	16	7.438±0.007	39.7±1.2	91.9±5.6	587.2±13.3	37.5±0.2	116.1±4.0
Sarin+PB	16	7.424±0.008	41.8±0.8	87.9±1.7	583.5±13.3	37.4±0.1	108.7±4.9

Table 1: Mean ± standard error of arterial blood pH, blood gases, body mass, body (rectal) temperature, and mean arterial blood pressure, recorded at the time of cerebral blood flow measurements. None of the means were significantly different from controls at the corresponding time after treatment, except for those of rectal temperature in the PB groups at 2 and 4 after treatment ($P < 0.05$, Bonferroni adjusted contrasts of three means against controls).

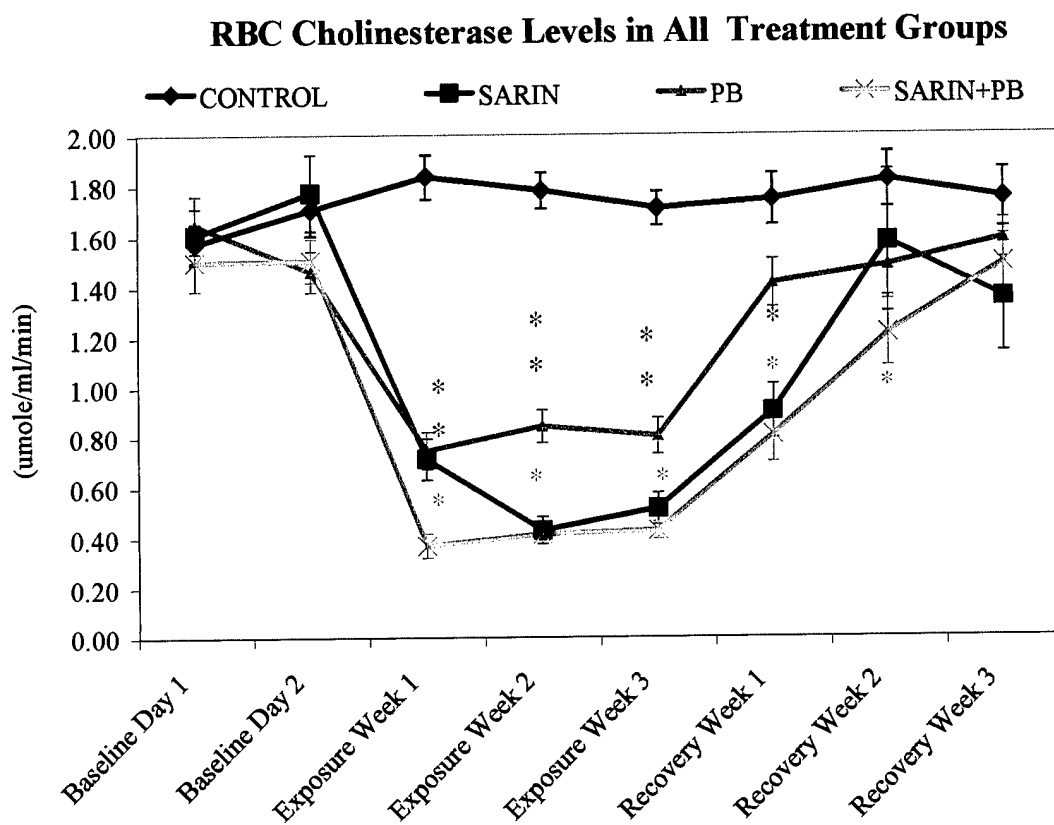


Figure 1: RBC ChE activity was measured before (Baseline), during treatment (Exposure weeks 1-3) and the immediate recovery period (Recovery weeks 1-3). Data (Means and SE) are in $\mu\text{moles/ml/min}$. * = significant vs. controls ($P < 0.05$).

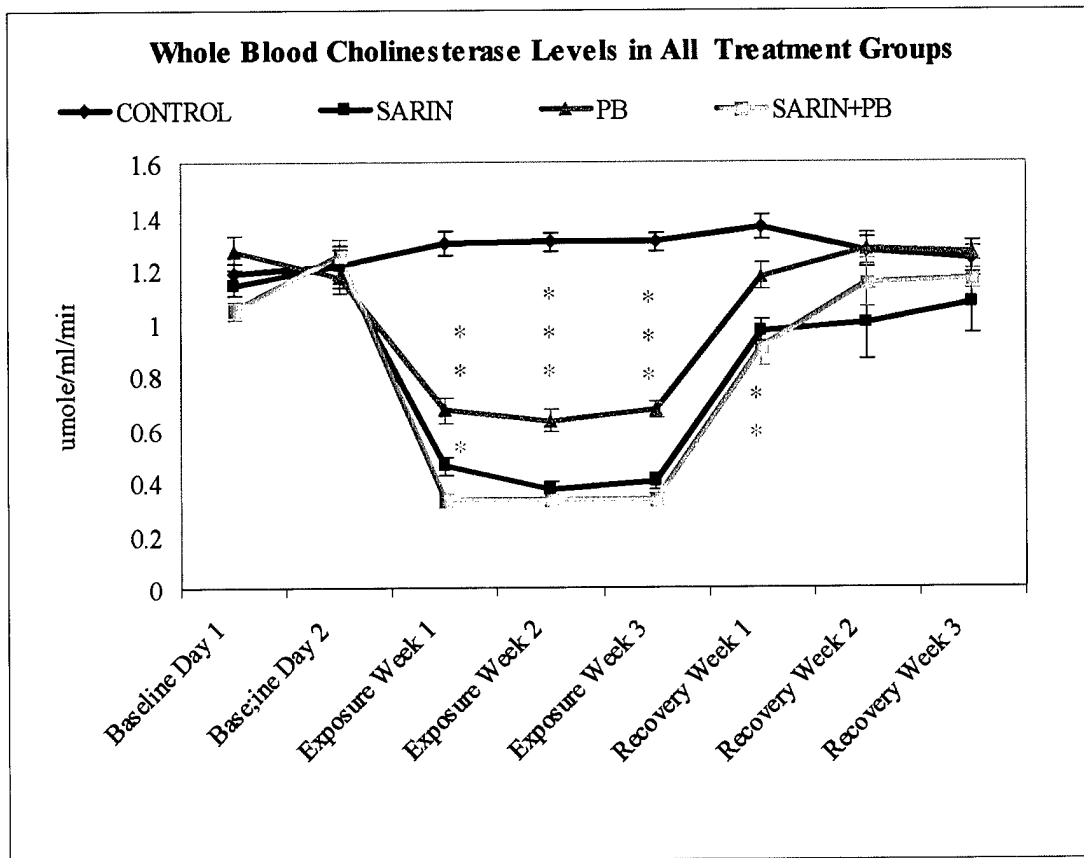


Figure 2: Whole blood ChE activity was measured before (Baseline), during treatment (Exposure weeks 1-3) and in the immediate recovery period (Recovery weeks 1-3). Data (Means and SE) are in $\mu\text{moles/ml/min}$. * = significant vs. controls ($P < 0.05$).

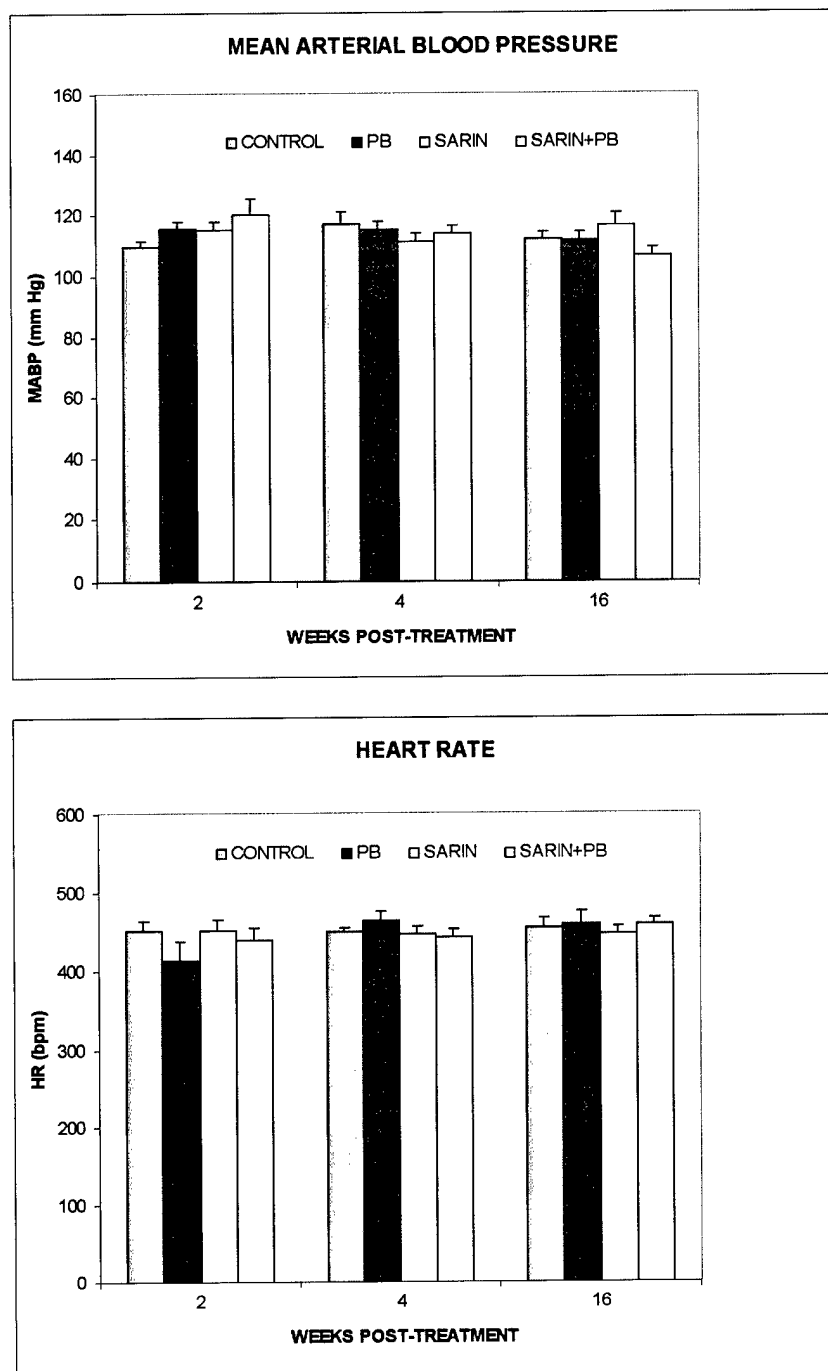


Figure 3: Mean and SE of resting mean arterial blood pressure (TOP), and heart rate (BOTTOM). No statistically significant differences among experimental groups were found.

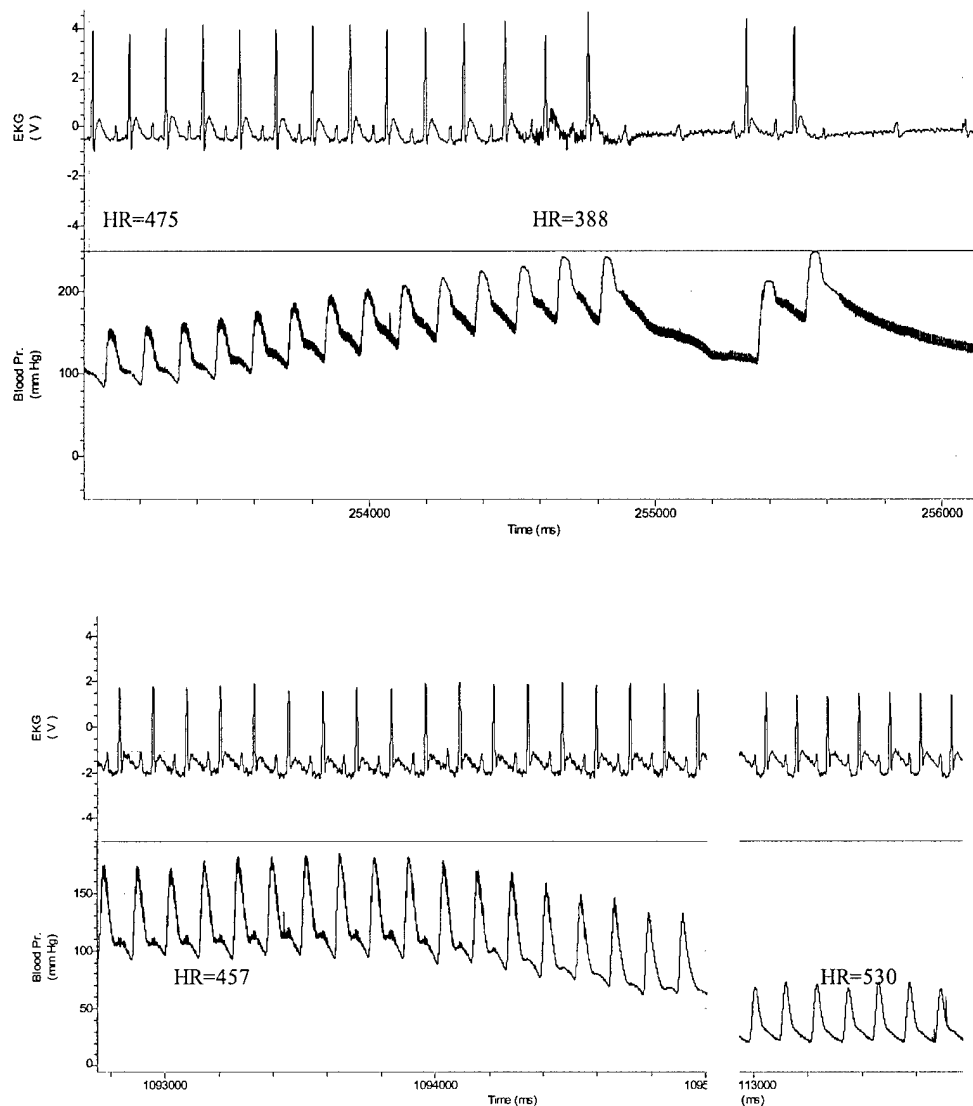


Figure 4: Representative baroreceptor mediated heart rate responses to pharmacologically induced hyper- or hypotension. TOP: Progressive hypertension and sinus bradycardia after phenylephrine (PE), followed by A-V block and nodal bygeminal rhythm. BOTTOM: Progressive hypotension and tachycardia following nitroprusside (NP). Two doses of each drug were given to every animal and the regression of HR on MABP calculated with or without inclusion of beats beyond the A-V block.

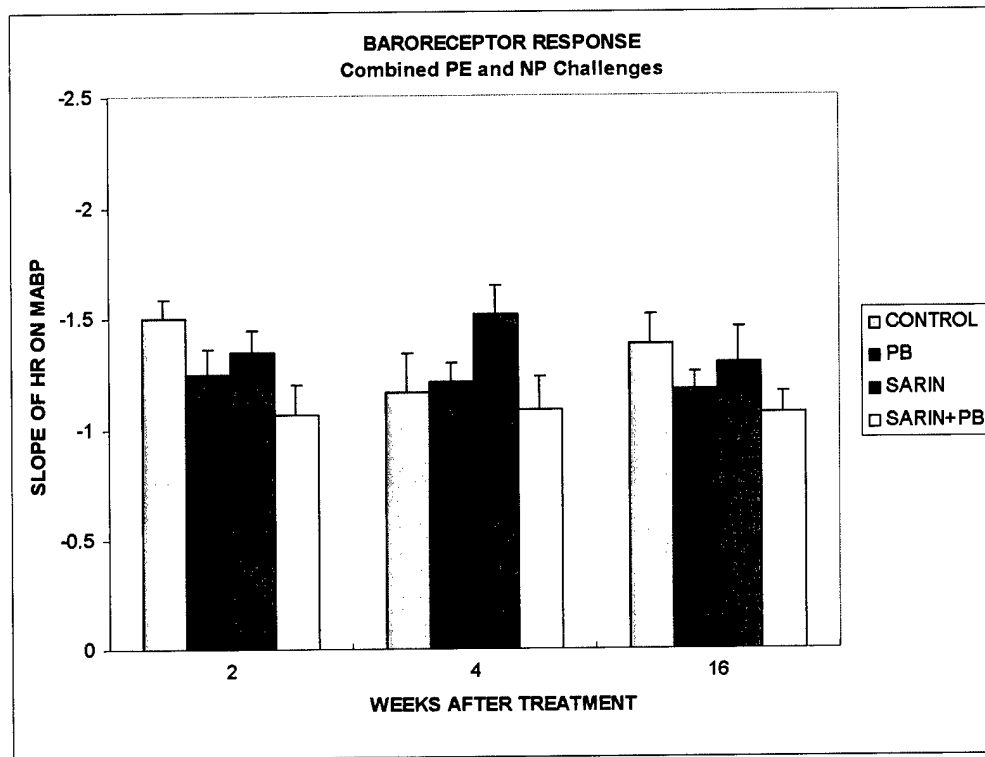


Figure 5: Mean and SE of slopes of the linear regression of HR on MABP for all PE and NP challenges excluding heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.

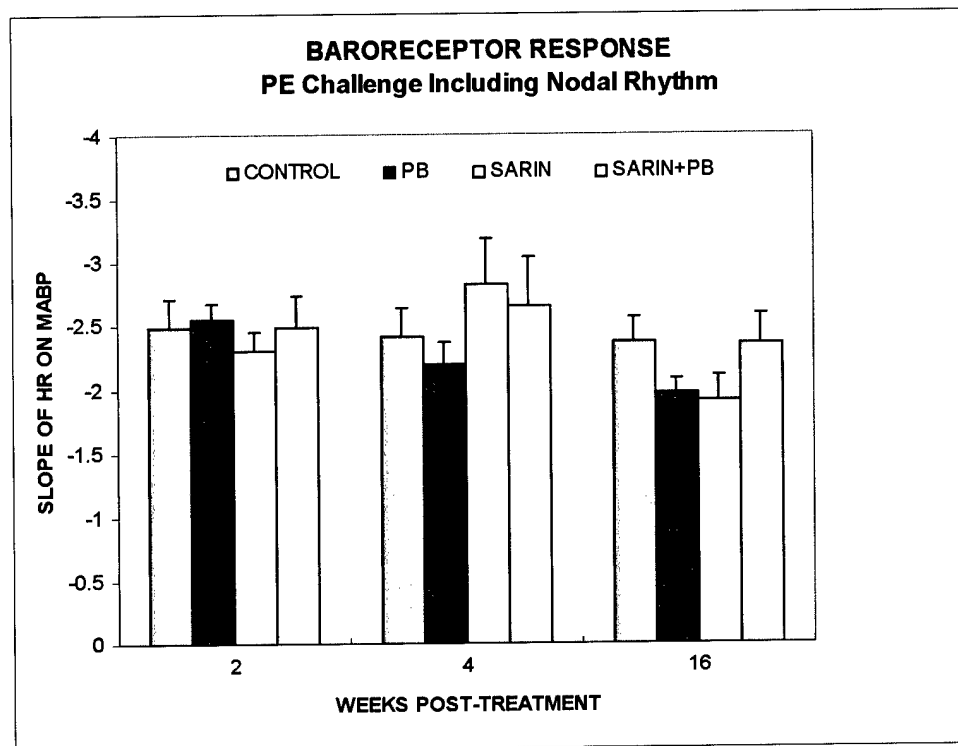


Figure 6: Mean and SE of slopes of the linear regression of HR on MABP for all PE challenges, including heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.

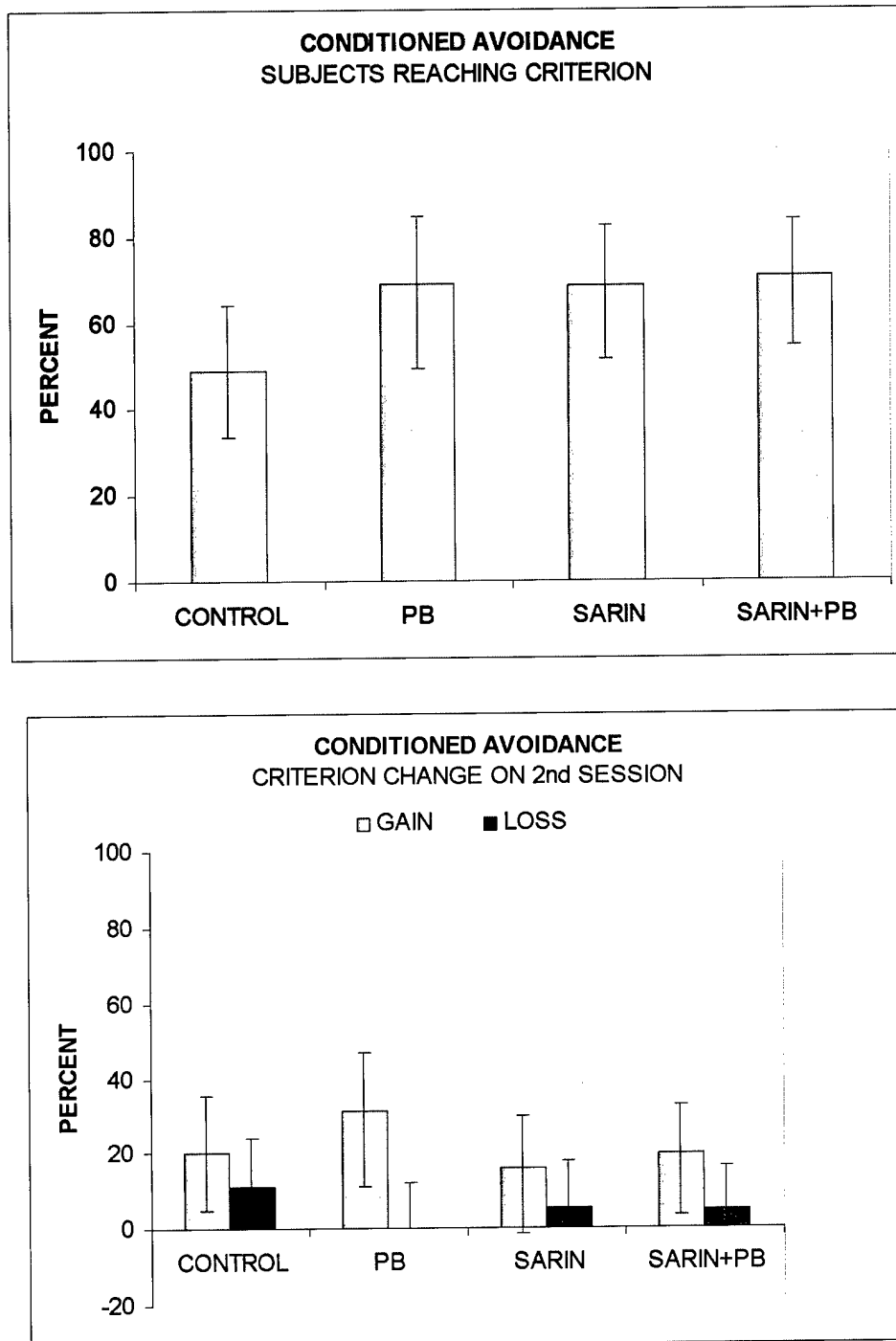


Figure 7: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test (top) and animals that gained or lost criterion in the second day when compared with the first (bottom). There were no statistically significant differences between groups (pooled data from all times after treatment).

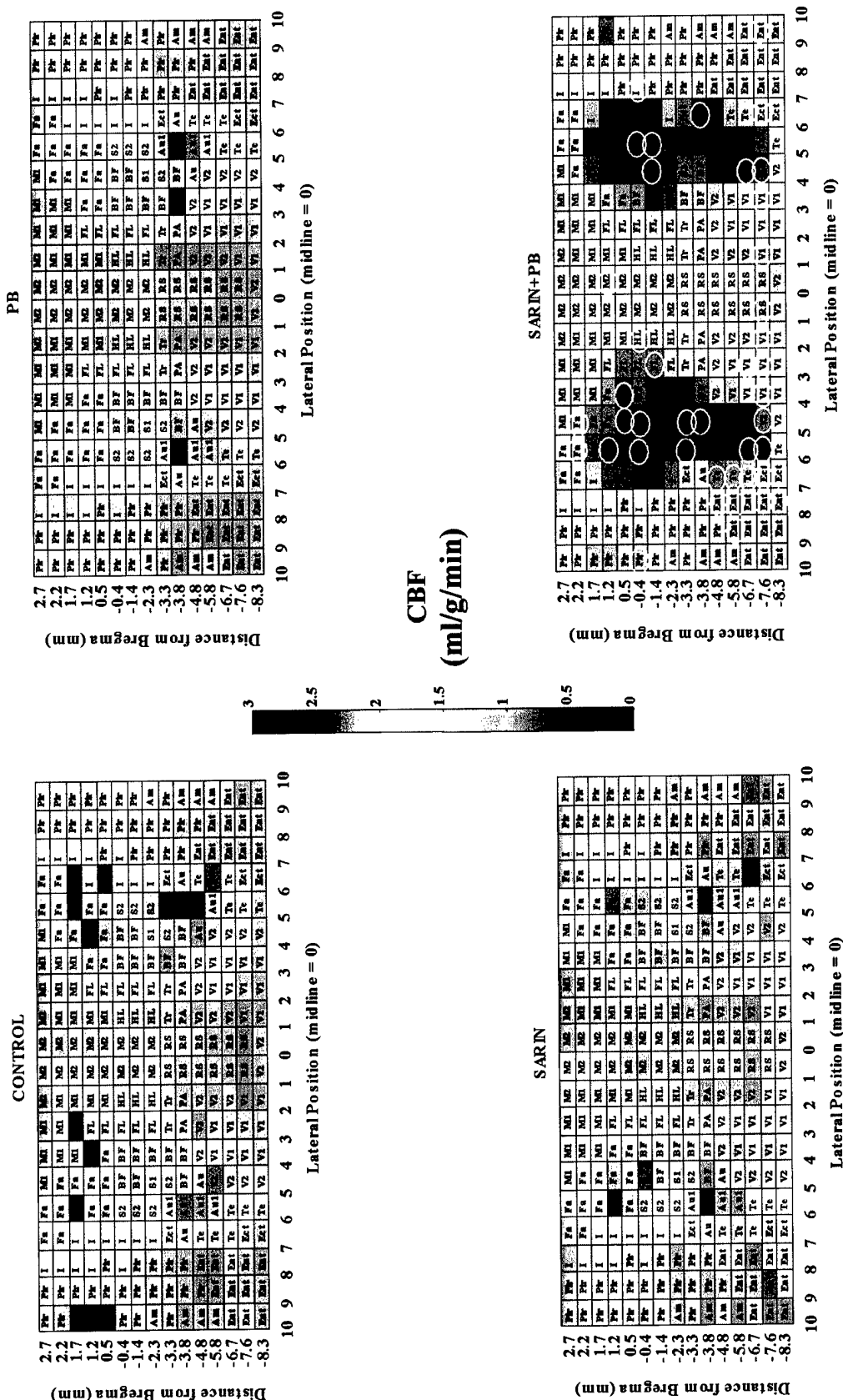


Figure 8: Cerebral blood flow (CBF) was measured with the Iodo-¹⁴C-antipyrine technique in 12 conscious rats per group, 2 weeks after treatment. 300 cortical regions were sampled in 15 coronal planes, identified by their distance from bregma in mm (ordinate), and their relative position from the midline (abscissa). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See text for abbreviations. Statistical significance against the control group is indicated by white ovals ($P < 0.05$, adjusted for three contrasts).

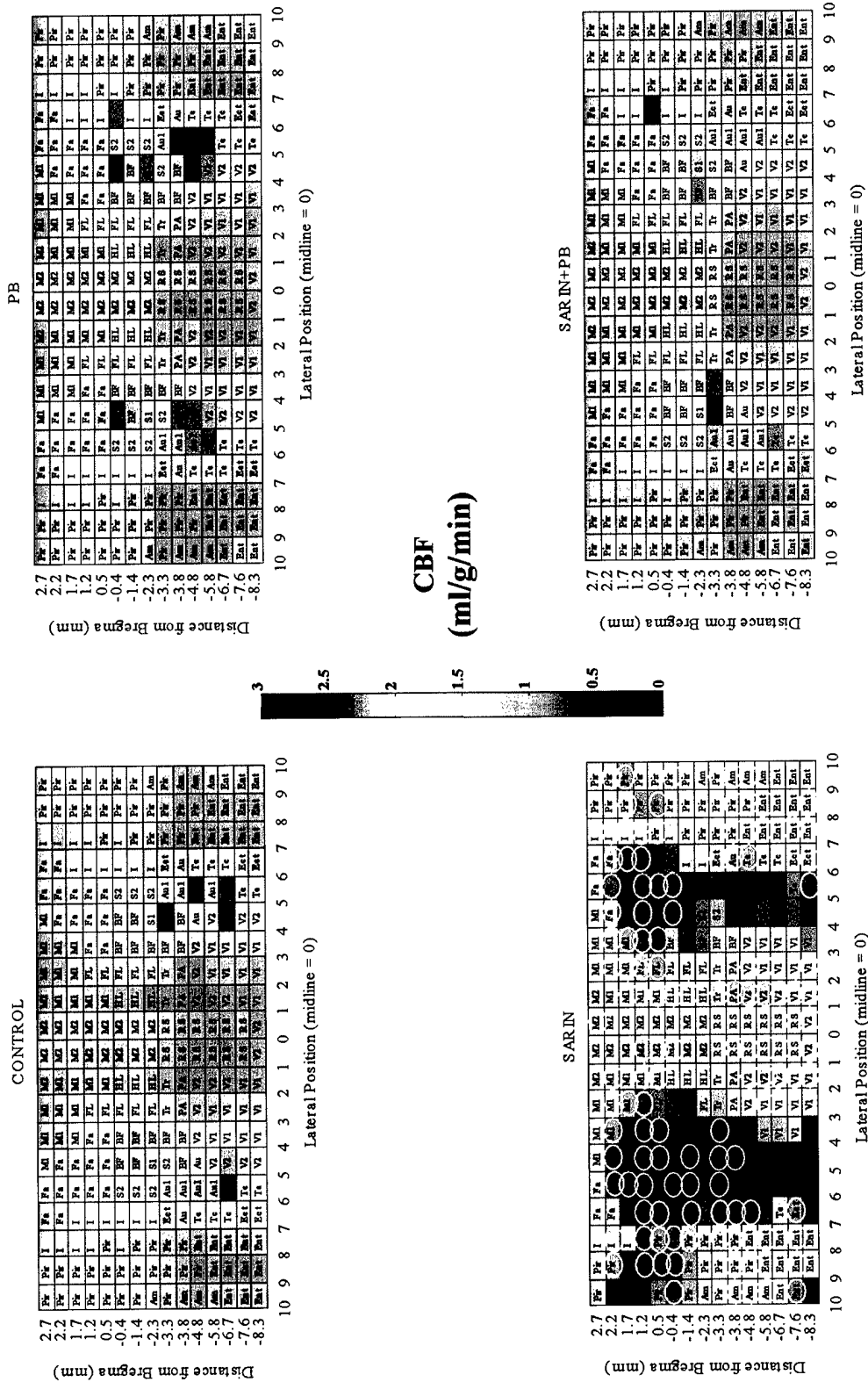


Figure 9: Cerebral blood flow (CBF) was measured with the Iodo-¹⁴C-antipyrine technique in 12 conscious rats per group, 4 weeks after treatment. 300 cortical regions were sampled in 15 coronal planes, identified by their distance from bregma in mm (ordinate), and their relative position from the midline (abscissa). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See text for abbreviations. Statistical significance against the control group is indicated by white ovals ($P < 0.05$, adjusted for three contrasts).

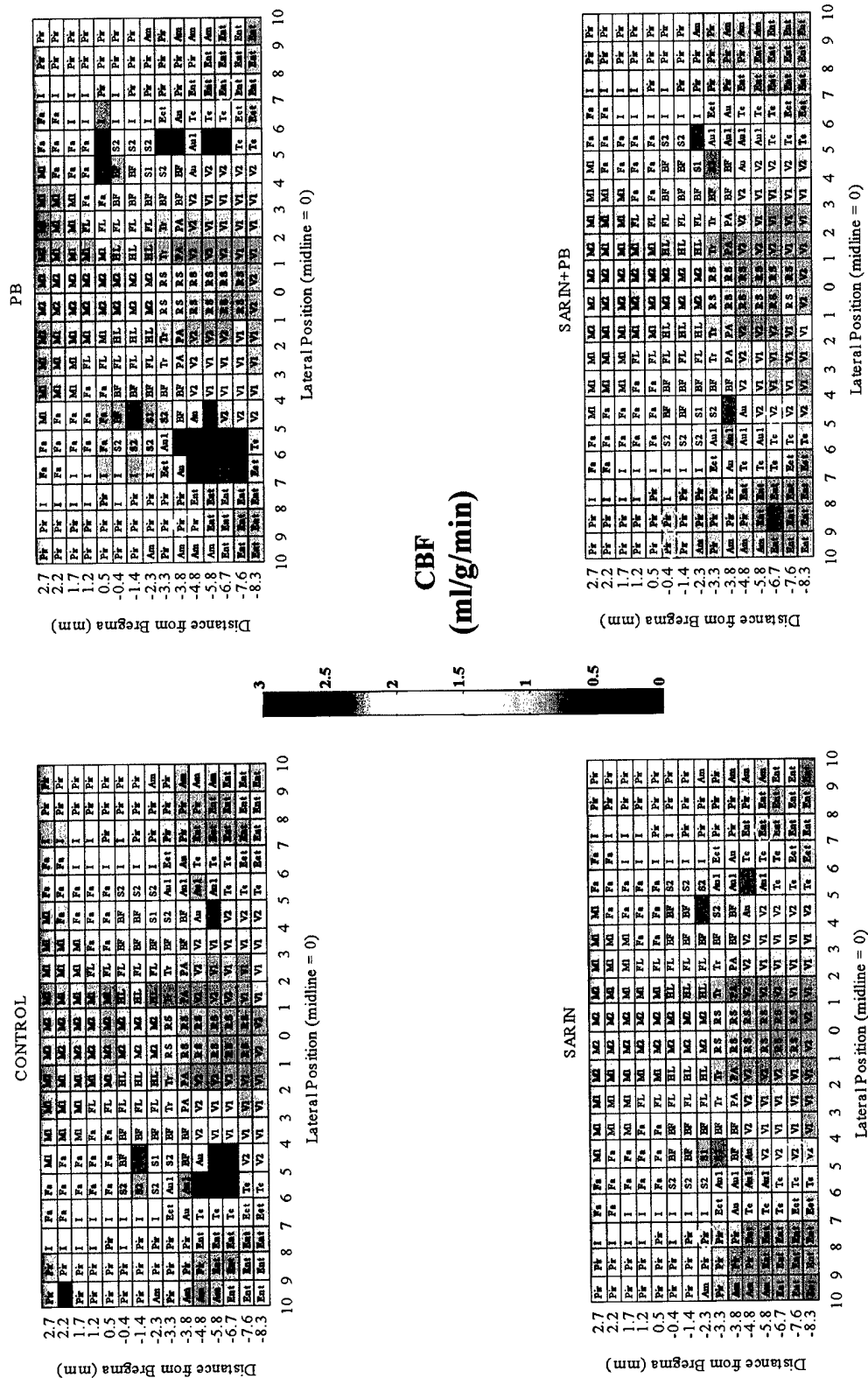


Figure 10: Cerebral blood flow (CBF) was measured with the Iodo-¹⁴C-antipyrine technique in 12 conscious rats per group, 16 weeks after treatment. 300 cortical regions were sampled in 15 coronal planes, identified by their distance from bregma in mm (ordinate), and their relative position from the midline (abscissa). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See text for abbreviations. Statistical significance against the control group is indicated by white ovals ($P < 0.05$, adjusted for three contrasts).

Abstract View

EFFECTS OF CHRONIC EXPOSURE TO LOW LEVELS OF CHOLINESTERASE INHIBITORS ON CEREBRAL BLOOD FLOW

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Cholinesterase (ChE) inhibitors are known to enhance cerebral blood flow (CBF) following acute administration. The effects of chronic administration of these agents have not been fully explored however. We exposed rats to the ChE inhibitors Sarin (organophosphorus, 0.5 LD50 s.c. 3 times/week) and pyridostigmine bromide (PB, a carbamate, 80 mg/L in drinking water) alone or in combination for 3 weeks. Controls received saline s.c. and drinking water without drug. At 2, 4 and 16 weeks after treatment, regional CBF was measured in conscious animals with the autoradiographic Iodo-[14C]-antipyrine method. Cerebral cortical CBF was determined in both hemispheres at 10 locations in each and at 19 coronal planes (380 locations per animal). Cortical maps of CBF showed, 2 weeks after discontinuation of treatment, a significant elevation in most neocortical regions of animals treated with the combination of PB and Sarin. Lower elevations in a limited number of locations were observed in animals treated with Sarin alone, and no changes were observed in those treated with PB alone. At 4 weeks after treatment, changes in animals treated with Sarin and Sarin+PB were reduced. Red blood cells ChE did not recover completely (33% inhibition remained) at 2 weeks post-treatment in the Sarin+PB group while the Sarin group was not statistically different from controls. This phenomenon may explain some differences in CBF between the groups.

Supported by: US Army MPMC DAMD17-00-2-0015.

Citation:

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EFFECTS OF CHRONIC EXPOSURE TO LOW LEVELS OF CHOLINESTERASE INHIBITORS ON CEREBRAL BLOOD FLOW

O.U. Scremin^{1,3}, T.M. Shih², L. Huynh², M. Roch¹, W. Sun¹, D. R. Chialvo^{1,3}, J. D'Elia², C. Cable² and D.J. Jenden⁴

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579.17

INTRODUCTION

• Carbamate cholinesterase inhibitors provide protection, when used as pretreatment, from exposure to organophosphorus cholinesterase inhibitors. The carbamate cholinesterase inhibitor pyridostigmine bromide (PB) was adopted by USA and NATO armies as wartime pretreatment and adjunct for nerve agent exposure. Large scale use of this pretreatment occurred during the Persian Gulf War, with relatively few side effects related to cholinergic hyperactivity in some subjects. This pretreatment, and the possible exposure to low level sarin, have been proposed to contribute to a conglomerate of symptoms experienced by Persian Gulf War veterans.

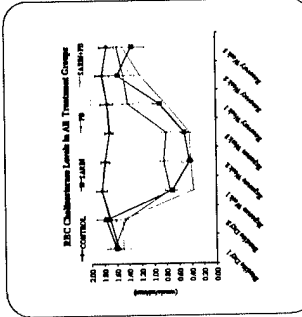
• This study is one of a series designed to determine if prolonged exposure to PB or low-dose sarin, alone or in combination, could elicit effects detectable after exposure to the agent.

• In the present experiments, cerebral blood flow (CBF) was measured at 2, 4, or 16 weeks after exposure to these agents, in order to detect possible effects of these drugs that may outlast their acute effects.

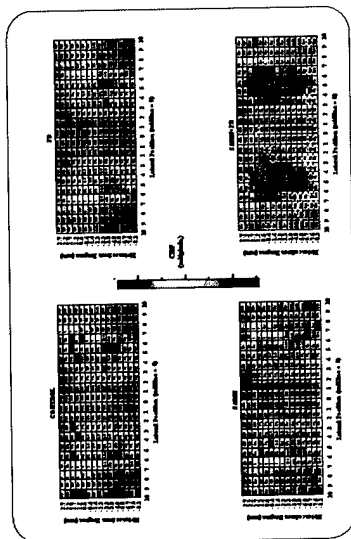
METHODS

- Adult Sprague-Dawley rats were used. Animals were treated for three weeks with either (1) 0.5% saline (s.c.) saline injection (CONTROL), (2) PB in drinking water (80 mg/L) (PB), (3) sarin 0.5 LD50 three times/week s.c. injection (SARIN), or (4) PB in drinking water plus sarin s.c. (SARIN+PB).
- Whole blood and RBC AChE activity, as well as plasma BuChE, were determined by an adaptation of the method of Ellman using the appropriate substrates.
- Following 2, 4 or 16 weeks after treatment, cerebral blood flow (CBF) was measured with the ¹⁴C-antipyrine autoradiographic technique in conscious animals. Measurements were performed in 300 regions of the cerebral cortex (10 locations on each hemisphere, in 15 coronal planes) on a total of 144 rats (12 rats/treatment x 4 treatments x 3 times after treatment).
- Arterial blood gases and pH, mean arterial blood pressure, rectal temperature, and body mass were recorded just prior to implementation of the CBF measurement procedure.

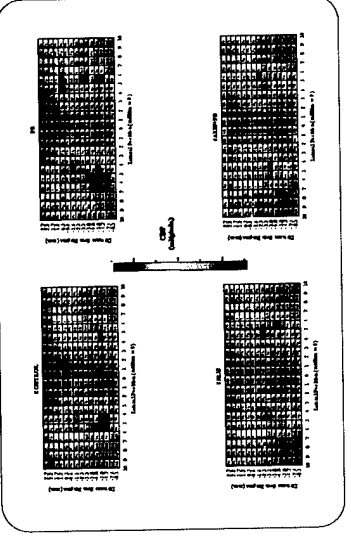
RESULTS



1. RBC AChE activity was measured before baseline, during treatment (Exposure weeks 1-3) and the immediate recovery period (Exposure weeks 4-6) for the experimental groups (n=12 rats/group). * significant vs. control (P<0.05).



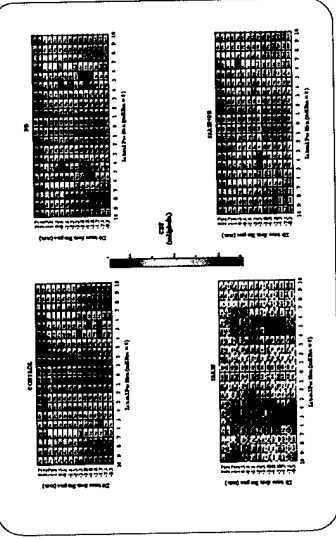
3. Cerebral blood flow (CBF) was measured with the ¹⁴C-antipyrine technique in 12 coronal planes in 300 regions of the cerebral cortex for the experimental groups (n=12 rats/group). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See Figure 3 for abbreviations.



5. Cerebral blood flow (CBF) was measured with the ¹⁴C-antipyrine technique in 12 coronal planes in 300 regions of the cerebral cortex for the experimental groups (n=12 rats/group). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See Figure 3 for abbreviations.

Treat	Weeks	Blood pH	pH CO ₂	Pa CO ₂	Body mass	Body temp	MAP
CONTROL	2	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
PB	2	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
SARIN	2	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
SARIN+PB	2	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
CONTROL	4	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
PB	4	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
SARIN	4	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
SARIN+PB	4	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
CONTROL	16	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
PB	16	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
SARIN	16	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4
SARIN+PB	16	7.40±0.03	38.4±0.3	38.4±0.3	460±10.3	37.5±0.2	110.0±4

2. Means and SE of arterial blood gases and pH, body mass, rectal temperature, and mean arterial blood pressure (MAP) for all experimental groups (12 rats per group). None of the means were significantly different from the corresponding means for the CONTROL group at 2, 4, or 16 weeks after treatment (P>0.05, Bonferroni adjusted contrasts of three means against controls).



4. Cerebral blood flow (CBF) was measured with the ¹⁴C-antipyrine technique in 12 coronal planes in 300 regions of the cerebral cortex for the experimental groups (n=12 rats/group). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See Figure 3 for abbreviations.

SUMMARY & CONCLUSIONS

- This study was designed to mimic the conditions of soldiers in the battlefield that are taking PB as a prophylactic treatment against nerve agents intoxication, with or without exposure to subasymptomatic levels of these agents.
- The results have shown that under these conditions, CBF was enhanced in many cortical locations, 2 and 4 weeks after treatment, in animals exposed to sarin+PB and sarin, respectively. No consistent changes were observed 16 weeks after treatment in any group.
- The effects on CBF observed at the shorter periods after treatment probably reflect residual cholinesterase inhibition in perivascular compartments.

Supported by US Army Medical Research and Materiel Command, DAMD 17-00 200015.

Abstract View

DELAYED EFFECTS OF CHRONIC EXPOSURE TO SUBTOXIC LEVELS OF CHOLINESTERASE INHIBITORS

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We exposed rats to subtoxic levels of the cholinesterase (ChE) inhibitors Sarin (0.5 LD₅₀ s.c. 3 times weekly) and pyridostigmine bromide (PB, 80 mg/L in drinking water) alone or in combination for 3 weeks. Controls received saline s.c. and drinking water without drug. At 2, 4 and 16 weeks after exposure, animals were tested for open field activity, auditory startle, passive avoidance, and nociceptive threshold. ChE and choline acetyltransferase (ChAT) activity were measured in somatosensory, temporal, and piriform cortex, hippocampus, striatum, thalamus, hypothalamus, mesencephalon, medulla, and cerebellum. No acute toxicity was found with any of the treatments. At 2 weeks, animals treated with sarin showed increase in auditory startle (Force units: Mean \pm SE, saline= 25.5 \pm 8.4, n=6; sarin= 54.3 \pm 6.5, n=10, P=< 0.05 vs Control, Fisher LSD; PB=32.9 \pm 5.9, n=12; sarin + PB= 25.4 \pm 5.9, n=12) and decrease in open field exploratory activity (distance walked in meters: saline= 43.5 \pm 2.4, n= 10; sarin= 31.0 \pm 2.2, n=12, P=< 0.01 vs Control; PB= 44.6 \pm 2.2, n=12; sarin + PB= 40 \pm 2.2, n=12). However, no differences were found at 4 and 16 weeks. There were no changes in regional ChE or ChAT between treatments. In conclusion, sarin-exposed rats showed decreased locomotor activity in the open field and enhanced auditory startle responses 2 weeks after exposure. These effects were not present in animals simultaneously treated with PB and sarin or at other times after exposure.

Supported by: US Army MPMC DAMD17-00-0015



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DELAYED EFFECTS OF CHRONIC EXPOSURE TO SUBTOXIC LEVELS OF CHOLINESTERASE INHIBITORS

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563.12

INTRODUCTION

• Carbamate cholinesterase inhibitors provide additional protection, when used as pretreatment, from exposure to soman and tabun than that afforded by atropine and oxime alone. On the basis of these findings, the quaternary cholinesterase inhibitor, pyridostigmine bromide (PB) was adopted by US military forces as a protective agent for soldiers exposed to nerve agents. The therapeutic target for this application of pyridostigmine has been to maintain inhibition of plasma butyrylcholinesterase (BuChE) between 20% to 40%. Large scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects. This pretreatment, and the possible exposure to low level sarin have been proposed to contribute to a conglomerate of symptoms experienced by Persian Gulf War veterans.

• The present study was designed to determine if exposure to PB or low-dose sarin, alone or in combination, could elicit a time when central and peripheral acetylcholinesterase (AChE) activity had returned to normal.

Methods

• Adult Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three week exposure period) and PB (the lowest protective dose). 50-300 mg/kg of sarin and 0.5-10 mg/kg of PB were tested. The decrease of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War.

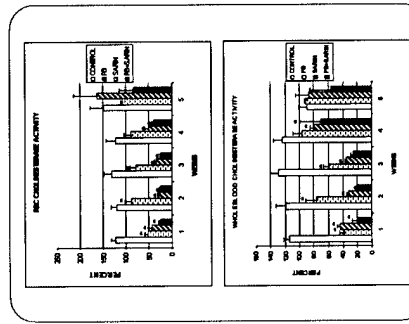
• Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates.

• Animals were treated during three weeks with either (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 mg/kg three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c. injection.

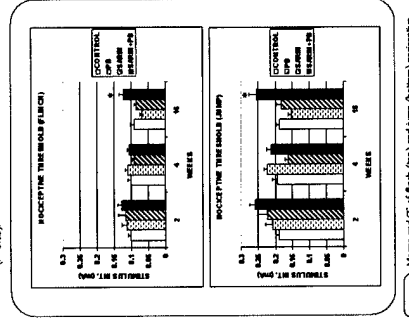
• After treatment, animals were tested for passive avoidance conditioning, nociceptive threshold, acoustic startle, and open field activity.

• After tests were completed, rats were euthanized and brain tissue regions of interest were microdissected from frozen brain slices. These regions were homogenized, and aliquots used for the determination of tissue AChE activity with the kinetic method of Ellman, and ChAT activity with the method of Fonnum.

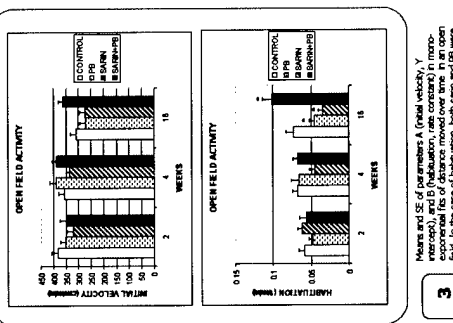
RESULTS



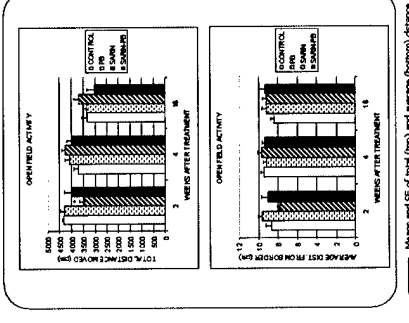
1. RBC (top) and whole blood (bottom) AChE activity was measured during three weeks of treatment. Data (means and SE) is percent of the average of two consecutive days (weeks 4-5). Data were significantly different from control (P < 0.05).



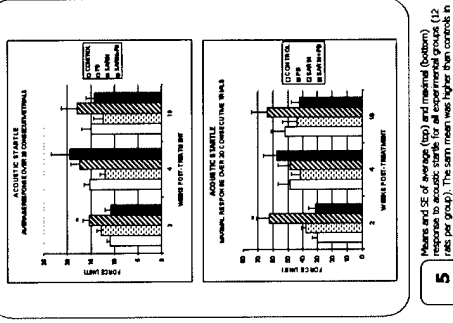
2. Means and SE of both (top) and (bottom) nociceptive threshold for all experimental groups (12 rats per group). The sarin-treated group was significantly lower than control (P < 0.05).



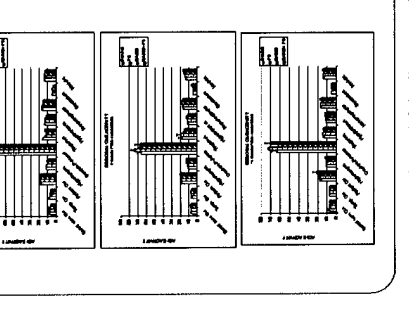
3. Means and SE of open field activity (top) and habituation time (bottom) over time. In an open field, rats were exposed to a novel environment and habituated to it. The mean habituation time was lower than control (P < 0.05) and lower than PB-treated rats (P < 0.05) while sarin-treated rats were significantly higher than all other groups (P < 0.001 vs sarin and PB, and P < 0.05 vs control).



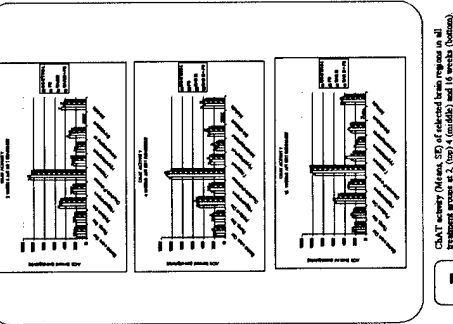
4. Means and SE of both (top) and average (bottom) distance moved in the open field for all experimental groups (12 rats per group). The sarin-treated group was significantly lower than control (P < 0.05).



5. Means and SE of acoustic startle response (top) and habituation time (bottom) over time. The mean habituation time was lower than control (P < 0.05) and lower than PB-treated rats (P < 0.05) while sarin-treated rats were significantly higher than all other groups (P < 0.001 vs sarin and PB, and P < 0.05 vs control).



6. AChE activity (means, SE) of selected brain regions in all treatment groups at 2, (top) 4 (middle) and 15 weeks (bottom). There were no significant differences between treatments.



7. ChAT activity (means, SE) of selected brain regions in all treatment groups at 2, (top) 4 (middle) and 15 weeks (bottom). There were no significant differences between treatments.

SUMMARY & CONCLUSIONS

• This study was designed to mimic the conditions of soldiers in the battlefield that are taking PB as a prophylactic treatment against nerve agents to intoxication, with or without exposure to subasymptomatic levels of these agents.

• The results have shown that under these conditions, PB did not produce adverse delayed neurobehavioral effects, other than a delayed elevation of nociceptive threshold. Moreover, simultaneous administration of PB and sarin prevented the development of decreased exploratory activity and enhanced responses to an acoustic startle test that were associated with sarin exposure without PB protection.

• Thus this study gives further support to the use of PB as one of the therapeutic resources against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level nerve agent exposure.

Supported by US Army Medical Research and Materiel Command, DAMD 17-00 200015.

Delayed effects of low-dose cholinesterase inhibitors on cardiovascular regulation

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Persian Gulf veterans complain of a conglomerate of symptoms that may be related to exposure to the cholinesterase inhibitors pyridostigmine bromide (PB) or sarin. To test if these agents could induce neurophysiological dysfunction, we exposed rats to subtoxic levels of Sarin (0.5 LD₅₀ s.c. 3 times weekly) and PB (80 mg/L in drinking water) alone or in combination for 3 weeks. Controls received saline s.c. and drinking water without drug. At 2, 4 and 16 weeks after exposure, the baroreceptor response (arterial blood pressure and heart rate changes to injection of phenylephrine or nitroprusside) and regional cerebral blood flow (rCBF, Iodo-[¹⁴C]antipyrine method) were assessed. No significant effects of treatments on the baroreceptors operation, or in the levels of arterial blood pressure and heart rate were found at any time after exposure. rCBF was enhanced in neocortex, entorhinal and ectorhinal cortex in the PB+sarin group at 2 weeks, and in the same regions plus piriform cortex in the sarin group at 4 weeks. No changes were observed 16 weeks after treatments. Thus, exposure of animals to PB and sarin induced only transient effects on cortical rCBF. Supported by US Army MRMCDAMD17-00-2-0015.

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DELAYED NEUROLOGIC AND BEHAVIORAL EFFECTS OF SUB-TOXIC DOSES OF CHOLINESTERASE INHIBITORS.

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- This work was supported by a contract from the US Army Medical Research and Materiel Command, DAMD17-00-2-0015.
 - Abbreviations: AChE= acetylcholinesterase; BuChE= butyrylcholinesterase; ChAT= cholineacetyltransferase; PB= pyridostigmine bromide.

ABSTRACT (246 words)

We tested the hypothesis that pyridostigmine bromide (PB) intake and/or low level sarin exposure, suggested by some as causes of the symptoms experienced by Persian Gulf War veterans, induce neurobehavioral dysfunction that outlasts their effects on cholinesterase. Adult male Sprague-Dawley rats were treated during three weeks with subcutaneous (s.c.) saline, PB in drinking water (80 mg/L), sarin, 62.5 µg/kg (0.5xLD50), three times/week s.c., or PB + sarin. Animals were tested for passive avoidance, nociceptive threshold, acoustic startle, and open field activity 2, 4 or 16 weeks after treatment.

Two weeks after sarin, acoustic startle was enhanced while distance explored in the open field decreased. These effects were absent with PB + sarin or PB by itself. No effect on any variable was found at 4 weeks, while at 16 weeks sarin induced a decrease and PB + sarin an increase in habituation in the open field test. Nociceptive threshold was elevated in the PB + sarin group at 16 weeks. No effect of treatment on passive avoidance was noted in any group. Brain regional AChE and ChAT activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon in the sarin group at 2 weeks.

In conclusion, this study gives further support to the use of PB against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level sarin exposure.

INTRODUCTION (631 words)

Many veterans of the Persian Gulf War complain from clusters of symptoms including cognitive alterations, balance disturbances and vertigo, and muscle aches and weaknesses (Haley, 2001), which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB), a carbamate, and/or sarin, a highly toxic organophosphorus (OP) chemical warfare nerve agent.

PB, like other carbamate ChE inhibitors, protects animals from the lethal effect of OP ChE inhibitors when given in anticipation of exposure to these OP agents. The mechanism of this protection appears to be the pre-occupation by the carbamate of ChE reactive sites, which become unavailable to the OP ChE inhibitor, with subsequent restoration of enzymatic activity due to the reversible decarbamylation of ChE. This phenomenon is the basis for the use of PB as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979; Leadbeater et al., 1985; Koplovitz et al., 1992; Kluwe et al., 1987; Keeler et al., 1991). The therapeutic target for this application of PB has been to maintain inhibition of plasma butyrylcholinesterase (BuChE) between 20% to 40%. Large scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). Possible exposure to sarin may have occurred following explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001).

The effects of low-level repeated exposure to OP nerve agents, not associated with acute clinical signs or symptoms, have attracted less attention than the well known effects of acute intoxication with these agents (Ecobichon and Joy, 1982; Sidell, 1974; Chambers, 1992) . Behavioral and electroencephalographic alterations in workers exposed to low levels (not associated with acute intoxication) of nerve agents have been reported (Burchfield and Duffy, 1982; Ecobichon and Joy, 1982). However, a study of human volunteers exposed to low to moderate levels of nerve agents has indicated no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer (Panel on Anticholinesterase Chemicals, 1982; Coordinating Subcommittee, 1985). The same conclusion appears to hold for low level accidental exposures to OP nerve agents (Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War veterans, could elicit cognitive or neurobehavioral abnormalities in experimental animals. Our initial experiments were aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment. This criterion was adopted because no episodes compatible with symptoms of acute intoxication with ChE inhibitors have been described in soldiers during the Persian Gulf War, although it is possible that low-level exposure to sarin may have occurred. In the case of PB, the optimal dose was defined as one producing 20-30% inhibition of plasma BuChE. This is the degree of BuChE inhibition reported for human subjects receiving the same PB

dosage as soldiers during the Persian Gulf War (Keeler et al., 1991) (90 mg PB over 24 hrs, divided in three oral doses).

Passive avoidance and open field activity tests were used to assess cognitive function and motor activity, respectively. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation, respectively, as well as the expression of muscarinic cholinergic receptors in the same animals that were subjected to the neurobehavioral tests mentioned above. Separate groups of animals were studied at 2, 4, or 16 weeks after 3 weeks of exposure.

MATERIALS AND METHODS.

Male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity ($50 \pm 10\%$) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and water were freely available. Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal

experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously (s.c.). PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a three-week period.

Determination of optimal doses of sarin, PB and their combination: A

preliminary verification of the LD50 of sarin in rats was conducted by the "up and down" method (Dixon, 1965) using 5 doses (3 animals per dose level) with 120 $\mu\text{g/kg}$ as the middle dose at intervals of 0.05 Log_{10} unit. To find the optimal dose for sarin, animals were administered LD50 doses of this agent in 0.1 unit increments starting from 0.2 and up to 0.7xLD50, three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of 6 animals per dose. The highest dose not associated with toxic signs (described in detail below) during this 3-week period was adopted for the main study.

After correction for surface area equivalence between rats and human subjects (Freireich et al., 1966), the PB rat dose equivalent to that used in humans during the Persian Gulf War was calculated as 9 mg/kg/day. Experiments were set up to measure the plasma butyrylcholinesterase (BuChE) activity as well as the possible existence of signs of cholinergic toxicity in animals receiving 2.5, 5, 10 or 20 mg/kg/day PB in the drinking

water during three weeks. Prior to this, the average daily drinking volume for the set of rats to be used (as ml of water intake per kg body mass per day) was determined by measuring volume of water consumption over a three-week period. This pilot study indicated that to achieve the desired daily doses described above, animals should be given PB in the drinking water at concentrations of 20, 40, 80, and 160 mg/L, respectively. The effects of PB treatment on plasma BuChE were monitored.

The optimal repeated dose of sarin to be used in combination with PB in drinking water at a concentration determined by the previous study was established as follows. While taking PB in drinking water, animals were administered doses of 0.3, 0.4, 0.5 or 0.6 LD₅₀ sarin s.c., three times (Mondays, Wednesdays, and Fridays) a week for three weeks in groups of 6 animals per dose.

Experimental groups: Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (Control group). Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline (PB group). Group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5xLD₅₀) (Sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above. (PB + sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 hour. At 09:00 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per

week. PB in drinking water was terminated and switched to regular tap water at 17:00 hour on Friday of the third week. Animal dosing procedures were performed at the USAMRICD laboratory. After a period of 1, 3, or 15 weeks following treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB + sarin treatments.

Number of animals was 12 per group, and the total number of groups (treatments x times after treatments) was also 12 with a grand total of 144 rats.

Observation of signs of intoxication: Animals were observed for signs of cholinergic intoxication for at least one hour following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano, 1988).

Blood ChE measurements: When animals were received at the USAMRICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein (Liu et al., 1999) on two separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at

about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period.

Blood was collected into an Eppendorf 1.5 mL microtube containing 50 μ L (1000 USP unit per ml) heparin sodium and mixed. Forty μ L of whole blood were transferred to another microtube containing 160 μ L 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off, and 20 μ L RBC's was transferred into a microtube containing 180 μ L 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at -75°C until ChE analysis.

Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman (Ellman et al., 1961) using acetylthiocholine and butyrylthiocholine as substrates, respectively.

Regional brain activity of ChAT and AChE, and QNB binding: Animals were euthanized by decapitation under deep halothane anesthesia (2.5% in 30% O₂ balanced with N₂O). The brain was rapidly removed and flash frozen in methylbutane cooled to -70°C . Brain regions were microdissected from frozen brain slices for the following ten anatomical locations in each animal: somato-sensory, temporal, and pyriform cortex, hippocampus, caudate-putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These tissue samples were homogenized, and aliquots of these homogenates

were used to determine tissue AChE activity with the kinetic method of Ellman (Ellman et al., 1961), ChAT activity with the method of Fonnum (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura and Snyder, 1974).

Inhibited (passive) avoidance response: This was measured in a "step through" apparatus (McGaugh, 1972), consisting of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel, and (c) a shock delivery unit adjustable for the intensity and duration (1 mA, 0.5 sec) of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hrs. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment lead immediately to the closing of a door and administration of footshock. Retention was tested after a 48-hr delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as "retention," a measure of memory of the single training session. The retention trials were set at a limit of 10 min.

Open field locomotor activity: This was measured during a 20-min session in circular open field chambers of 60 cm diameter, with walls 45 cm high, under low level red light illumination. This is done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a Sony CCD video camera

(sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc., The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Distance traveled was summated at 1-min intervals, and these values were fitted by non-linear regression, using the Marquardt algorithm, to the model:

$$Y = A \cdot e^{-Bt} \quad (1)$$

where Y = distance moved (cm) and t = time after initiation of test (min). The values of parameters A (initial velocity, cm min⁻¹) and B (habituation, min⁻¹) were obtained as described above for every animal. Analysis of variance (ANOVA) was then performed for the two parameters using factors treatment (control, PB, sarin and PB + sarin) and time after treatment (2, 4, or 16 weeks).

In addition, total distance traveled and mean distance to the arena's border (the inner surface of the chamber's wall) during the entire test were also calculated for every animal.

Reactivity (startle response): Reactivity is defined as a response to a sudden brief and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been previously described (Silverman et al., 1988; Russell and Macri, 1979). In this procedure the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom-designed computer

program delivers a controlled sound and integrates and digitizes the movement-related electrical signal. Quantification of the response is provided in arbitrary force units. In the currently reported experiments, 20 trials were performed at fixed intervals of 10 seconds.

Nociceptive threshold: The procedure to measure nociceptive threshold used in these experiments has been described (Crocker and Russell, 1984) and utilizes reaction to a mild electric foot shock as its measure. It involves the "up and down" method (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 sec at 10-sec intervals. The shock intensities were available in a range from 0.05 mA to 0.4 mA and arranged in a \log_{10} scale at 0.1 \log_{10} units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animal's response on each trial. A "flinch" was defined as an elevation of 1 or 2 paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data is presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each interval after exposure to drugs or saline with one factor (treatment) at four levels (control, PB, sarin, PB + sarin). This was followed, if significant

(probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

RESULTS.

Dose Finding Studies: The LD50 of sarin was determined to be 125 µg/kg, sc. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/L had inhibition of plasma BuChE slightly greater than 20% on average. This was within the target effect set for these experiments (20 to 30% inhibition). The next higher PB concentration in drinking water (160 mg/L) induced a larger plasma BuChE inhibition (between 27 and 40 %). Thus, the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No sign of toxicity, as defined in Methods, was found in animals drinking water containing PB during three weeks.

The dose finding for sarin and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose that did not caused observed acute toxic effects when given alone or in combination with PB (80 mg/L in drinking water).

Body mass: Means of body mass, recorded daily during weekdays, through the three weeks of treatment and the subsequent two weeks following treatment are shown in Fig 1. No statistically significant difference was found between treatments. The expected increase in body mass with age was observed at the beginning of the experiments that

assessed outcome variables (2, 4 or 16 weeks after treatment), but no difference among treatment groups was found at these time points either.

Blood ChE activity: Measurements of RBC AChE during the 3 drug treatment weeks, the pre-treatment week (two measurements) and 3 post-treatment weeks are shown in Fig 2. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the three weeks of treatment. Sarin and PB + sarin produced an average decrease in RBC AChE to 35% and 27% of pre-treatment respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Nociceptive threshold : Data are presented in Fig. 3 for both the flinch and jump responses.

Flinch response: No statistically significant difference among groups was found for the flinch response to the test at 2 or 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher LSD test, $P < 0.05$) showed that the nociceptive threshold of the animals that received the combination of PB + sarin (0.117 ± 0.011 mA) was significantly higher than all other groups (controls= 0.091 ± 0.012 mA; PB= 0.068 ± 0.010 mA; sarin= 0.086 ± 0.012 mA).

Jump response: ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response

was significantly lower in the sarin group (0.17 ± 0.017 mA) than in the PB (0.23 ± 0.017 mA) and PB + sarin (0.211 ± 0.016 mA) groups, but not significantly different from controls (0.19 ± 0.016 mA). At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the PB+sarin group had a significantly higher threshold (0.255 ± 0.016 mA) than all other groups (controls= 0.18 ± 0.017 mA; PB= 0.152 ± 0.016 mA; sarin= 0.17 ± 0.018 mA)

Open field locomotor activity:

Parameter A (initial velocity): No statistically significant difference among treatments was found at 2 or 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for PB + sarin (360.6 ± 19.9 cm min⁻¹) was significantly higher than for the PB (272.8 ± 19.9 cm min⁻¹) group and sarin (275.3 ± 20.8 cm min⁻¹) group but not different from controls (309.5 ± 20.8 cm min⁻¹) (Fig 4).

Parameter B (habituation): No statistically significant difference among treatments was found at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter means for sarin (0.035 ± 0.0088 min⁻¹) group and PB (0.046 ± 0.0084 min⁻¹) group were lower than for controls (0.072 ± 0.0093 min⁻¹) while PB + sarin (0.101 ± 0.0084 min⁻¹) was significantly higher than all other groups (Fig. 4).

Total distance moved: ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (3451 ± 207 cm) was significantly lower than controls (4328 ± 338 cm) (Fig. 5). No difference vs. controls was found for the

other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

Distance to arena's border: ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and PB + sarin (9.05 ± 0.45 cm), but not different from controls (8.63 ± 0.64 cm) (Fig. 5).

Reactivity (acoustic startle): A significant increase in the average motor response in sarin-treated animals (15.3 ± 1.14 F.U.) against the controls (10.9 ± 1.14 F.U.) over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (sarin = 62.6 ± 5.49 F.U.; controls 30.0 ± 5.49 F.U.; PB = 37.7 ± 5.02 F.U.; PB + sarin = 31.1 ± 5.01 F.U.). In this case, the mean of the sarin group was significantly higher than of all others. No difference among group means was present at 4 or 16 weeks after treatment (Fig. 6).

Passive avoidance: No difference between experimental groups was found in the time to enter the dark compartment 24 hrs after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response (data not shown).

Brain regional ChE activity: Areas rich in cholinergic nerve cells and terminals were found to have, as expected, the highest ChE activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-

treatment time points studied (Table 1). Central ChE activity was not significantly modified with respect to controls at the time of measurements of tested variables. Sarin-treated animals studied at the end of outcome variables evaluation had evidently recovered from central ChE inhibition. This is in agreement with the substantial recovery of blood ChE activity recorded for this group at about the same time after treatment (Fig.2).

Brain regional ChAT activity: Areas rich in cholinergic nerve cells and terminals were found to have, as in the case of ChE, the highest ChAT activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (Table 2).

Brain regional QNB binding: Two weeks after treatment, there was a generalized decrease in QNB binding of the sarin group, when compared with controls, that was statistically significant in caudate-putamen, hippocampus and mesencephalon (Table 3). No statistically significant changes from control were found at 4 or 16 weeks post-treatment in this or any other group.

DISCUSSION (1210 words).

Previous experimentation has shown that some functions can be affected at levels of nerve agents (such as soman and sarin) below the threshold for clinical toxicity (Chippendale et al., 1972; Russell, 1982; Wolthuis and Vanwersch, 1984). Repeated low-level exposures to soman (0.3 LD₅₀) in rats induces initial decreases in body

temperature, temporal perception, and locomotor activity. Tolerance was observed to all these effects, except soman-induced hypoalgesia. No effect of soman on memory was found by these authors (Russell et al., 1986). In another study, animals treated with low-level soman (0.4 LD50), and followed up to 6 weeks while in the treatment regime, exhibited a hyper-reactivity condition (Shih et al., 1990). In none of these cases were animals studied beyond the period of drug administration. Effects of low-dose soman on an equilibrium test in rhesus monkeys were reported to wear off 24 hrs after exposure (Switzer et al., 1990). Exposure to low dose sarin has been recently reported to induce a decrease in activity and mobility, alteration of gait, and increase in stereotyped behavior and excitability in rats that persisted 3 to 12 months (Kassa et al., 2001a), as well as a deficit in Y-maze performance that subsided three weeks after exposure (Kassa et al., 2001b).

In the present series, the initial experiments were successful in finding reproducible effects on plasma BuChE activity of a PB concentration of 80 mg/L in the drinking water, with an estimated dose of about 10 mg/kg body mass/day. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for prophylaxis of OP poisoning (1.2 mg/kg body mass/day), based on surface area dosage conversion (Freireich et al., 1966). The degree of plasma BuChE inhibition obtained with this dose was within the range reported for humans taking 90 mg of PB orally per 24 hrs, divided in three doses (Keeler et al., 1991).

Sarin, and PB + sarin produced more pronounced and stable inhibition of RBC AChE than did PB. AChE inhibition recovered completely by the end of the second week after discontinuation of treatment for all groups. Animals did not show signs of acute toxicity during or following treatment. The conditions established for this experimental model, i.e., exposure to the highest dose of sarin, alone or in combination with PB, devoid of acute toxicity, were thus met.

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test two weeks after the discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Russell et al., 1986; Overstreet, 1977). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease particularly pronounced in caudate-putamen, hippocampus and mesencephalon. Down regulation of muscarinic receptors may have played a role in the behavioral phenomena described above since this was their only neurochemical correlate.

No effect of PB on locomotor activity was found. An earlier report (Hoy et al., 1999) had indicated a decrease in locomotor activity in rats given PB, but this effect was observed immediately after treatment with doses higher than used in the present study.

Both the depressed locomotor activity and enhanced reactivity induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from sarin lethality (Harris and Sticher, 1984).

Previous experimentation (Servatius et al., 1998) has reported a delayed enhancement of the acoustic startle response, with lower doses and shorter exposure times of PB than those reported here, in Wistar-Kyoto, but not Sprague-Dawley rats. The Wistar-Kyoto rats in those experiments were reported to have a basal plasma BuChE activity 27% lower than the Sprague-Dawley rats. These authors speculated that this fact might have caused a greater penetration of PB into the central nervous system, on account of the diminished scavenging effect of BuChE, and by that mechanism mediated the exaggerated acoustic startle response. In our experiments, we have used a dose almost ten times higher than the lower dose at which Servatius et al. reported enhancement of acoustic startle, for a longer period of time (21 days as opposed to 7), but we still did not observe any effects of PB on this response. In fact, as stated above, PB protected sarin treated animals from the delayed behavioral effects (decreased locomotor activity and hyper-reactivity) of sarin administration.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990; Russell et al., 1986), and the reverse is true of hypercholinergic states (Shih and Romano, 1988). A delayed elevation of nociceptive threshold for both the flinch and the jump response was found in the animals that had received PB + sarin, a phenomenon most

clearly demonstrated 16 weeks after treatment. These results are difficult to interpret in the light of current knowledge of ChE inhibitors effects on pain, since no central ChE inhibition was detected at this late time. These intriguing findings deserve further exploration with other methodologies for pain threshold evaluation.

The lack of changes in the passive avoidance paradigm indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. Possible cognitive effects of the three treatments will be tested at later stages of this project by two other learning paradigms, conditioned avoidance response and Morris water maze. Learning impairments have been previously described in rats receiving PB (Liu, 1992; Shih et al., 1991). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (10 mg/kg/day), equivalent, on the basis of body surface area conversion between species, to that taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day). Moreover, in the two earlier studies referenced above, behavioral tests were performed within minutes of dosing, with no long-term follow up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of acute symptomatology, but assessment was limited to the period immediately following treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, this study was designed to mimic the conditions of soldiers in the battlefield that are taking PB as a prophylactic treatment against nerve agents intoxication, with or without exposure to sub-symptomatic levels of these agents. PB was

administered in the drinking water so as to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans. The results have shown that under these conditions, PB did not produce adverse delayed neurobehavioral effects. Moreover, at 2 weeks post-treatment, simultaneous administration of PB and sarin prevented the development of decreased exploratory activity and enhanced response to an acoustic startle test that were associated with sarin exposure without PB protection. Thus, this study gives further support to the use of PB as one of the therapeutic resources against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level nerve agent exposure. Further experimentation is planned to determine the possible effects of this treatment protocol on other physiological and neurobehavioral parameters.

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LEGENDS FOR FIGURES

FIGURE 1: Body mass was recorded daily (except on weekends) during the three weeks of drug treatment and the following 3 weeks. Data are averages of all animals in each experimental group: 144 rats for the first 4 weeks and 96 rats for the last 2 weeks. No statistically significant difference between groups was found.

FIGURE 2: RBC AChE during the 3 drug treatment weeks (T1-3), the pre-treatment week (two measurements, Pre 1-2) and 3 post-treatment weeks (Post 1-3). Data (Means and SE) are expressed as $\mu\text{moles/ml/min}$. ANOVA was significant for the 3 weeks of treatment and the first week post-treatment, and multiple comparisons (Fisher LSD test, $P < 0.05$) indicated that all groups were different from controls (indicated as * in figure) in all those four conditions.

FIGURE 3, Top panel: Means and SE of flinch nociceptive threshold for all experimental groups used (12 rats per group). ANOVA was significant at 16 weeks after treatment and multiple comparisons (Fisher LSD test, $P < 0.05$) indicated that the sarin+PB mean was significantly higher than controls (indicated as * in figure) and all other treatments at that time. **Bottom panel:** Means and SE of jump nociceptive threshold for all experimental groups used (12 rats per group). ANOVA was significant at 4 weeks and 16 weeks. At 4 weeks, multiple comparisons indicated that the sarin mean was lower than the PB and sarin+PB means, but not different from controls. At 16 weeks after treatment, the sarin+PB mean was significantly higher than controls (indicated as * in figure) and all other groups.

FIGURE 4: Means and SE of parameters A (initial velocity) and B (habituation) in non-linear fits of open field exploratory activity for all groups (12 rats per group). **Top panel:** ANOVA was significant for initial velocity and multiple contrasts (Fisher LSD test, $P < 0.05$) indicated that sarin + PB was significantly higher than the PB and the sarin groups but not different from controls. **Bottom panel:** ANOVA was significant only at week 16 for habituation, and multiple contrasts indicated that sarin and PB alone were lower than controls (indicated as * in figure), while sarin+PB was significantly higher than controls and all other groups.

FIGURE 5, Top panel: Means and SE of total distance moved (cm) during the open field test for all experimental groups used (12 rats per group). ANOVA was significant only at 2 weeks after treatment. Multiple contrasts (Fisher LSD test, $P < 0.05$) indicated that the sarin mean was significantly lower than controls at that time (indicated as * in figure). **Bottom panel:** Means and SE of average distance (cm) from the circular arena border for all experimental groups used (12 rats per group). Distance to arena's border: ANOVA was significant only at 2 weeks after treatment. Multiple contrasts indicated that the sarin mean was significantly lower than PB, and sarin+PB, but not different from controls.

FIGURE 6, Top panel: Means and SE of average acoustic startle response across trials for all experimental groups used (12 rats per group). ANOVA was significant at 2 weeks only. Multiple comparisons (Fisher LSD test, $P < 0.05$) indicated that the sarin mean was higher than controls (indicated as * in figure). **Bottom panel:** ANOVA was also significant only at 2 weeks for the maximal response over 20 consecutive trials. The sarin mean was higher than controls and all other groups.

Table 1: Acetylcholinesterase activity (nanomoles/mg tissue/min). Data shown are mean \pm S.E. of 12 animals per experimental condition and time post-treatment

<i>2 weeks post-treatment</i>				
	Control	PB	Sarin	Sarin+PB
Somat sens Ctx	7.8 \pm 0.5	7.4 \pm 0.3	7.2 \pm 0.7	6.3 \pm 0.2
Temporal Ctx	7.7 \pm 0.3	7.0 \pm 0.2	7.0 \pm 0.3	5.9 \pm 0.5
Piriform Ctx	17.9 \pm 1.0	18.8 \pm 1.4	18.4 \pm 1.5	17.7 \pm 1.3
Hippocampus	11.5 \pm 0.5	11.6 \pm 0.2	10.3 \pm 0.7	10.7 \pm 0.6
Caudate-Putamen	73.5 \pm 3.1	68.7 \pm 3.4	67.0 \pm 4.4	67.1 \pm 4.3
Thalamus	15.7 \pm 0.6	12.5 \pm 0.7	11.3 \pm 0.7	12.7 \pm 1.0
Hypothalamus	13.0 \pm 1.2	12.3 \pm 1.0	12.9 \pm 0.8	10.8 \pm 0.7
Mesencephalon	16.2 \pm 1.1	16.5 \pm 0.6	15.0 \pm 1.1	15.7 \pm 0.5
Cerebellum	4.4 \pm 0.2	4.1 \pm 0.3	4.4 \pm 0.3	3.8 \pm 0.3
Medulla	13.4 \pm 0.7	13.4 \pm 0.7	13.2 \pm 0.8	12.3 \pm 1.2
<i>4 weeks post-treatment</i>				
	Control	PB	Sarin	Sarin+PB
Somat sens Ctx	7.3 \pm 0.3	7.0 \pm 0.1	7.4 \pm 0.2	7.1 \pm 0.3
Temporal Ctx	7.0 \pm 0.4	7.2 \pm 0.1	8.1 \pm 0.3	7.0 \pm 0.4
Piriform Ctx	18.6 \pm 1.2	18.2 \pm 1.3	18.5 \pm 0.8	19.3 \pm 1.2
Hippocampus	10.6 \pm 0.8	12.0 \pm 0.2	12.4 \pm 0.6	12.4 \pm 0.4
Caudate-Putamen	66.0 \pm 4.4	74.1 \pm 3.4	66.7 \pm 2.9	67.2 \pm 6.3
Thalamus	18.3 \pm 3.8	19.2 \pm 5.4	15.9 \pm 0.7	14.8 \pm 0.7
Hypothalamus	10.9 \pm 0.9	11.8 \pm 0.3	12.9 \pm 0.5	11.2 \pm 0.4
Mesencephalon	16.5 \pm 0.5	17.2 \pm 0.6	17.9 \pm 0.8	17.2 \pm 0.4
Cerebellum	4.6 \pm 0.2	4.8 \pm 0.1	4.3 \pm 0.4	6.2 \pm 1.2
Medulla	12.9 \pm 1.2	14.2 \pm 0.4	15.7 \pm 0.4	15.9 \pm 1.0
<i>16 weeks post-treatment</i>				
	Control	PB	Sarin	Sarin+PB
Somat sens Ctx	7.6 \pm 0.9	7.4 \pm 0.2	7.3 \pm 0.5	7.2 \pm 0.2
Temporal Ctx	7.6 \pm 0.4	7.3 \pm 0.1	6.6 \pm 0.2	6.8 \pm 0.2
Piriform Ctx	15.8 \pm 1.9	18.3 \pm 0.7	17.3 \pm 0.8	20.9 \pm 1.7
Hippocampus	10.0 \pm 0.8	10.4 \pm 0.3	10.0 \pm 0.5	10.8 \pm 0.7
Caudate-Putamen	63.4 \pm 5.4	71.4 \pm 1.7	61.3 \pm 2.6	70.2 \pm 2.3
Thalamus	12.7 \pm 1.0	13.9 \pm 0.5	12.7 \pm 0.4	11.9 \pm 0.9
Hypothalamus	10.7 \pm 0.4	11.0 \pm 0.3	11.1 \pm 0.6	10.2 \pm 0.6
Mesencephalon	15.9 \pm 0.9	16.5 \pm 0.3	14.4 \pm 1.1	16.3 \pm 0.4
Cerebellum	5.1 \pm 0.5	4.5 \pm 0.1	4.3 \pm 0.2	4.7 \pm 0.1
Medulla	13.2 \pm 0.5	13.7 \pm 0.4	12.8 \pm 0.7	13.4 \pm 0.4

Table 2: Choline acetyltransferase activity (ACh formed, $\mu\text{moles/g/hr}$). Data shown are mean \pm S.E. of 12 animals per experimental condition and time post-treatment

2 weeks post-treatment								
	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	5.57	± 0.47	5.93	± 0.33	5.33	± 0.49	5.24	± 0.33
Temporal Ctx	6.46	± 0.49	5.75	± 0.22	5.66	± 0.38	5.38	± 0.51
Piriform Ctx	14.52	± 0.68	13.11	± 0.79	13.32	± 1.26	12.25	± 1.20
Hippocampus	7.34	± 0.60	6.95	± 0.24	7.37	± 0.57	6.13	± 0.79
Caudate-Putamen	29.21	± 2.62	28.57	± 1.62	29.56	± 2.36	27.67	± 2.74
Thalamus	6.60	± 0.92	6.49	± 0.35	7.09	± 0.57	7.07	± 0.89
Hypothalamus	6.11	± 1.48	4.76	± 0.40	4.24	± 0.64	3.99	± 0.66
Mesencephalon	8.94	± 0.81	8.32	± 0.64	8.00	± 0.77	6.78	± 0.51
Cerebellum	0.81	± 0.11	0.80	± 0.04	0.79	± 0.03	0.65	± 0.09
Medulla	11.20	± 1.62	12.96	± 1.16	13.84	± 2.68	10.41	± 1.83
4 weeks post-treatment								
	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	4.95	± 0.51	5.15	± 0.15	5.95	± 0.22	4.15	± 0.56
Temporal Ctx	5.59	± 0.46	5.76	± 0.22	6.67	± 0.27	4.58	± 0.82
Piriform Ctx	13.68	± 1.53	13.74	± 0.70	15.18	± 0.61	14.22	± 1.71
Hippocampus	6.20	± 0.71	7.81	± 0.23	7.78	± 0.24	7.12	± 0.99
Caudate-Putamen	28.04	± 2.84	29.80	± 0.75	30.38	± 1.21	27.02	± 3.40
Thalamus	7.46	± 0.64	6.65	± 0.25	8.25	± 0.34	6.62	± 0.79
Hypothalamus	4.60	± 1.01	3.39	± 0.15	6.06	± 0.87	3.27	± 0.49
Mesencephalon	7.80	± 0.62	7.59	± 0.30	9.78	± 0.41	7.06	± 0.82
Cerebellum	0.65	± 0.08	0.80	± 0.04	0.89	± 0.08	0.66	± 0.07
Medulla	11.42	± 1.22	10.12	± 0.80	11.80	± 1.20	10.04	± 1.96
16 weeks post-treatment								
	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	6.57	± 0.64	6.76	± 0.10	4.82	± 0.65	5.11	± 0.27
Temporal Ctx	6.57	± 0.42	7.39	± 0.24	6.10	± 0.15	5.71	± 0.21
Piriform Ctx	15.71	± 1.37	16.40	± 0.43	13.85	± 0.91	13.79	± 0.68
Hippocampus	8.50	± 0.41	8.95	± 0.24	8.13	± 0.25	7.43	± 0.27
Caudate-Putamen	30.85	± 2.57	35.55	± 1.23	27.99	± 1.00	28.28	± 1.23
Thalamus	6.87	± 0.84	9.89	± 1.13	6.76	± 0.39	5.98	± 0.32
Hypothalamus	5.24	± 1.37	4.38	± 0.21	4.09	± 0.33	3.36	± 0.16
Mesencephalon	7.33	± 0.96	8.63	± 0.21	7.04	± 0.70	7.18	± 0.43
Cerebellum	1.58	± 0.75	0.81	± 0.04	0.87	± 0.05	0.79	± 0.05
Medulla	12.12	± 1.67	11.92	± 1.27	12.48	± 1.15	10.37	± 1.21

Table 3: 3H-QNB binding (fmol/mg tissue). Data shown are mean \pm S.E. of 12 animals per experimental condition and time post-treatment.

2 weeks post-treatment								
	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	132.4	± 9.5	125.4	± 6.1	112.2	± 11.2	114.6	± 10.8
Temporal Ctx	125.4	± 5.0	125.6	± 4.2	96.7	± 14.5	105.6	± 7.5
Piriform Ctx	121.8	± 7.9	107.5	± 2.8	93.2	± 12.9	98.0	± 5.9
Hippocampus	115.9	± 3.5	114.4	± 4.7	92.2	± 10.5*	95.7	± 6.6
Caudate-Putamen	177.8	± 12.9	171.1	± 7.6	128.8	± 15.7*	158.2	± 12.1
Thalamus	68.6	± 3.5	61.6	± 1.5	60.1	± 8.4	53.9	± 4.2
Hypothalamus	42.1	± 5.0	38.3	± 1.4	29.3	± 4.4	36.5	± 3.0
Mesencephalon	48.9	± 2.3	42.9	± 1.7	32.5	± 4.4*	44.3	± 3.6
Cerebellum	9.9	± 0.7	10.4	± 1.0	6.2	± 1.2	9.4	± 1.3
Medulla	36.4	± 1.6	35.7	± 1.4	36.7	± 8.1	35.8	± 3.0
4 weeks post-treatment								
	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	125.9	± 7.2	123.9	± 3.9	131.9	± 5.2*	107.8	± 6.9
Temporal Ctx	121.1	± 6.4	123.7	± 2.7	121.3	± 10.3	106.1	± 6.0
Piriform Ctx	111.7	± 4.6	111.7	± 3.2	110.9	± 6.3	107.2	± 4.2
Hippocampus	105.4	± 5.7	118.0	± 3.2	107.9	± 8.3	105.8	± 6.3
Caudate-Putamen	170.4	± 6.5	182.0	± 4.5	187.5	± 9.7	160.2	± 6.2
Thalamus	64.1	± 3.1	61.4	± 1.8	58.8	± 3.0	60.1	± 3.3
Hypothalamus	33.3	± 2.4	39.4	± 1.8	39.5	± 1.8	36.0	± 3.5
Mesencephalon	51.3	± 3.0	44.3	± 1.5	48.1	± 1.7	45.4	± 3.2
Cerebellum	10.1	± 0.8	9.5	± 0.4	10.1	± 0.8	10.2	± 1.3
Medulla	36.9	± 3.5	35.1	± 2.3	41.2	± 2.5	43.0	± 3.4
16 weeks post-treatment								
	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	97.1	±9.2	108.3	±4.0	116.3	±11.6	101.4	±4.2
Temporal Ctx	113.3	±4.7	117.4	±3.5	118.8	±8.6	117.1	±5.0
Piriform Ctx	102.2	±2.6	108.1	±3.9	109.5	±7.9	95.6	±3.4
Hippocampus	109.2	±5.7	120.4	±5.2	109.9	±8.5	115.3	±2.4
Caudate-Putamen	159.8	±7.5	167.8	±3.2	157.1	±11.3	163.4	±7.7
Thalamus	56.8	±2.9	61.8	±2.1	56.6	±3.8	59.9	±3.4
Hypothalamus	37.1	±1.7	35.9	±1.8	38.7	±3.2	29.2	±1.1
Mesencephalon	36.5	±4.0	41.9	±0.8	45.4	±3.6	36.1	±1.2
Cerebellum	10.8	±2.2	7.9	±0.5	10.7	±1.0	9.5	±0.4
Medulla	30.4	±1.2	32.3	±1.2	37.1	±2.8	30.2	±1.6

* Statistically significant by ANOVA and Fisher's LSD tests.



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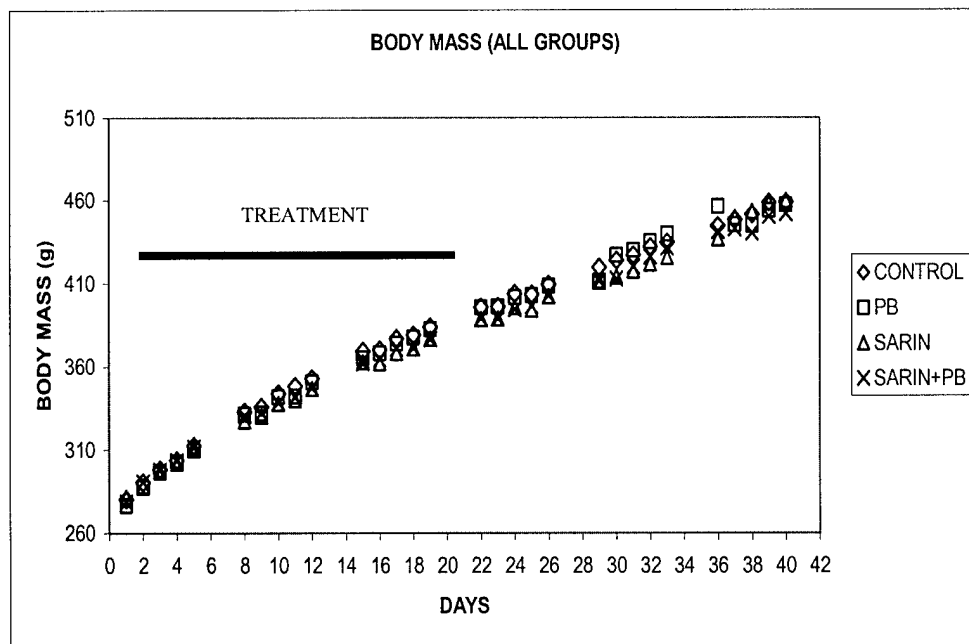


FIGURE 1

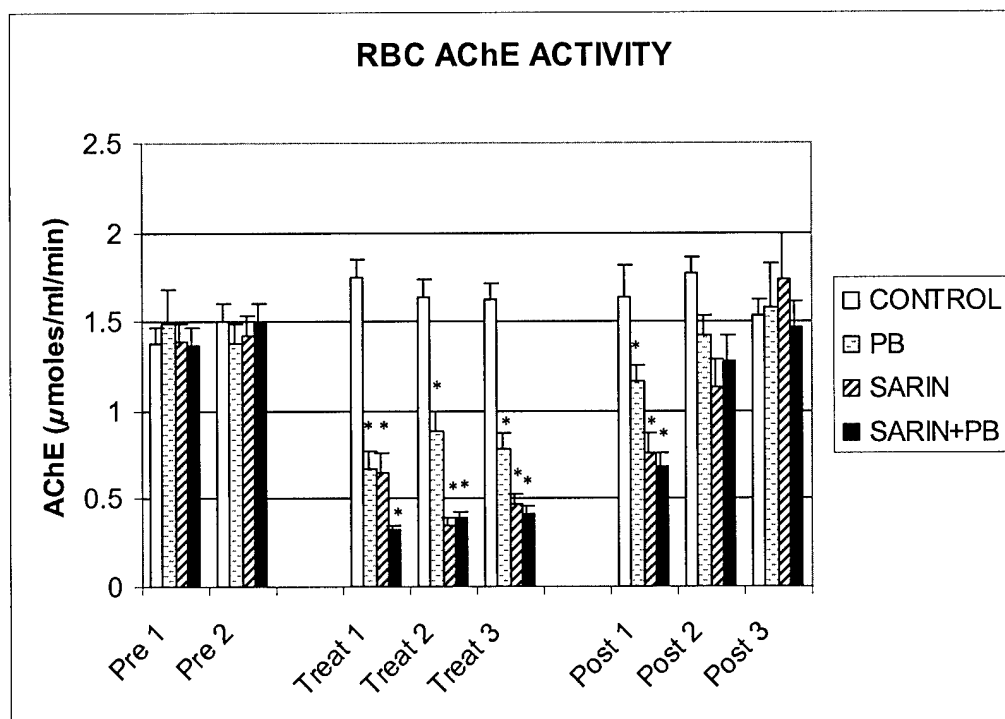


FIGURE 2

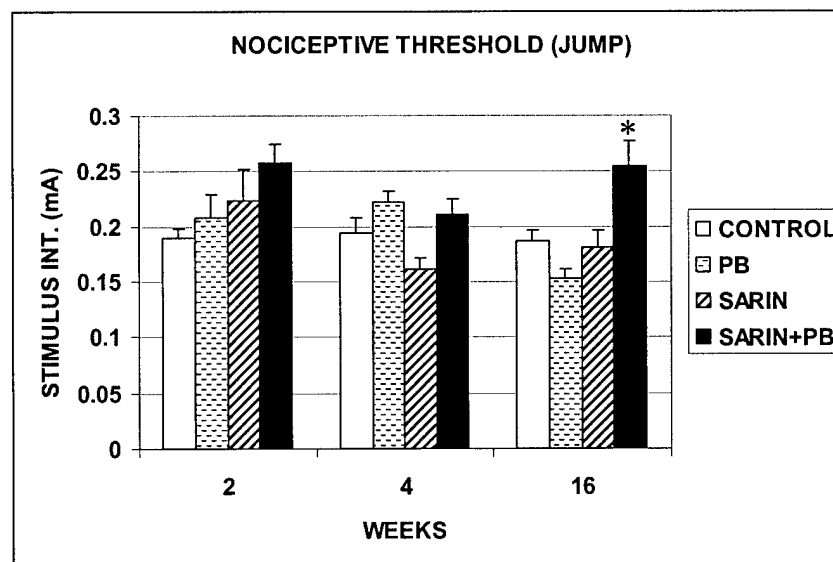
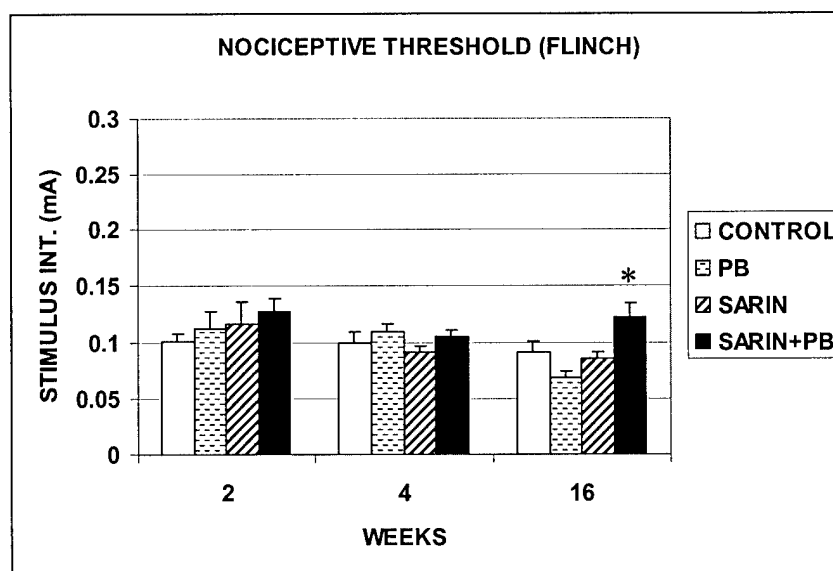


FIGURE 3

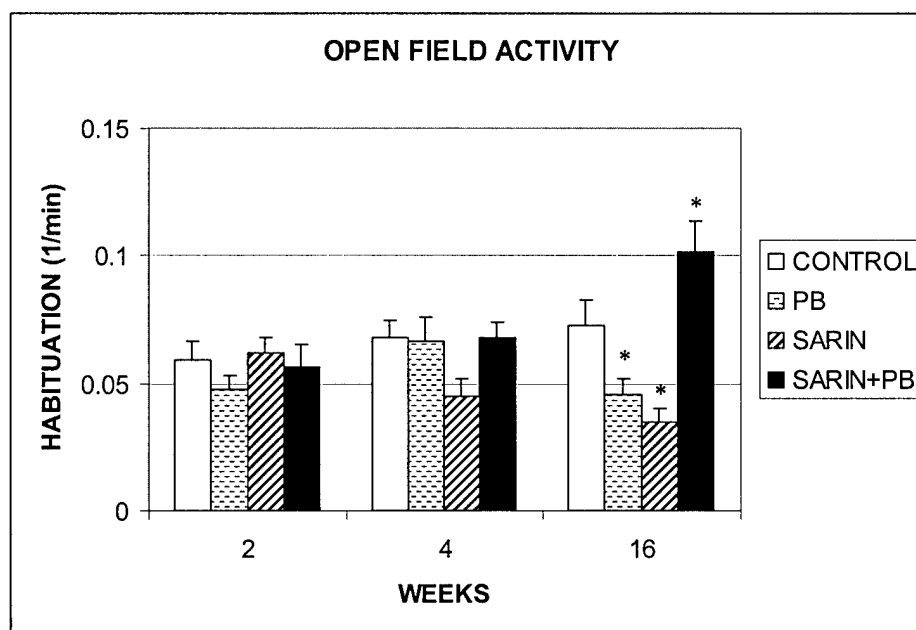
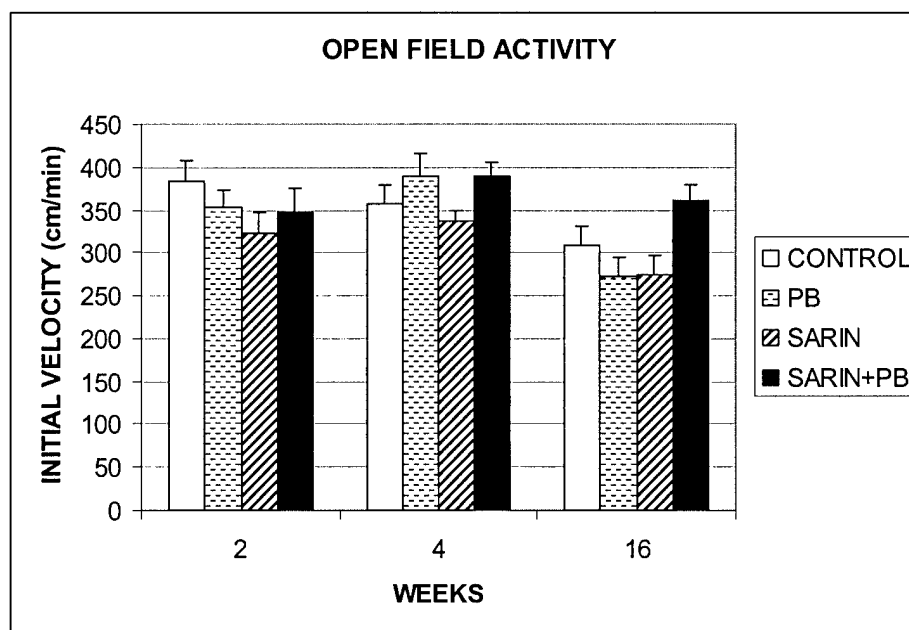


FIGURE 4

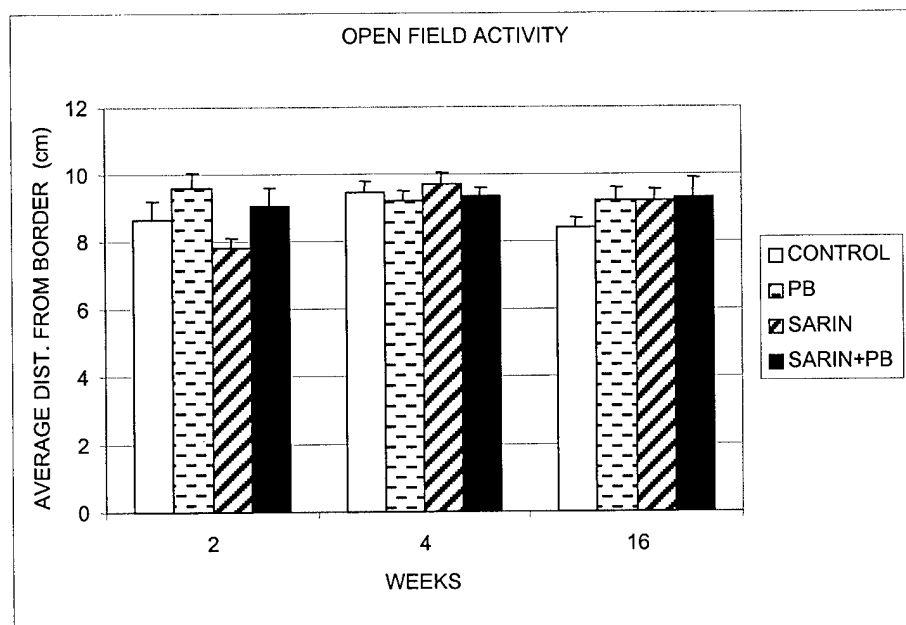
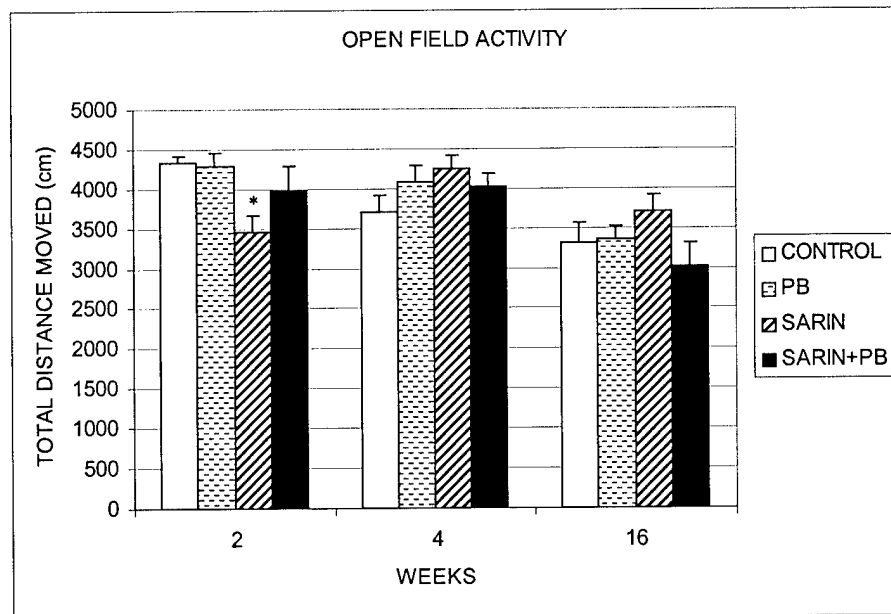


FIGURE 5

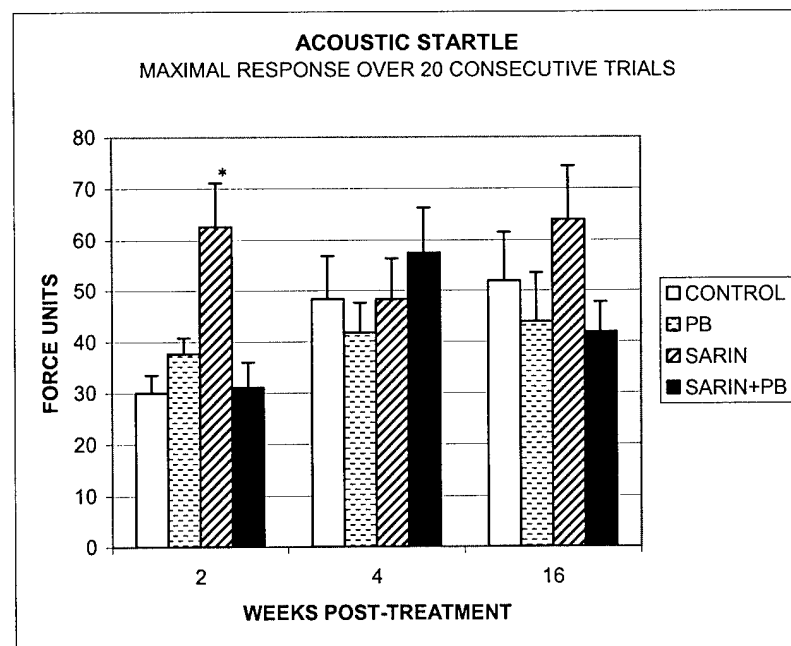
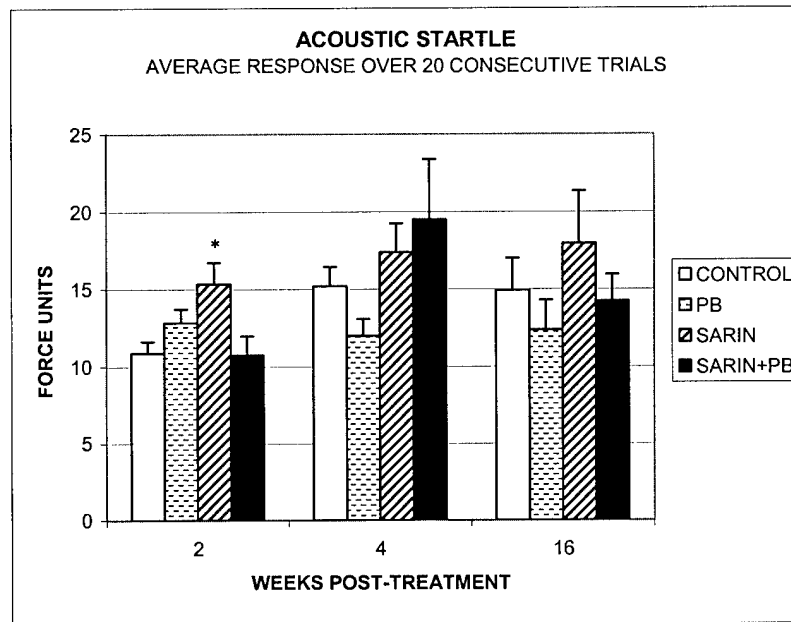


FIGURE 6

EFFECTS OF LOW-DOSE CHOLINESTERASE INHIBITORS ON COGNITION.

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Abstract

Veterans from the Persian Gulf War complain of neurological and cognitive dysfunction, ascribed by some authors to pyridostigmine bromide (PB) and/or sarin exposure. In the present experiments, passive (PA) and conditioned (CA) avoidance learning and habituation (HAB) of exploration of a novel environment were used to assess cognition in male Sprague-Dawley (CrI:CD(SD)IGSBR) rats at 2, 4, and 16 weeks after exposure to non-toxic doses of PB and sarin alone or in combination. Measured parameters were retention time 48 hrs after conditioning (PA), criterion (6 consecutive avoidances), escape and avoidance time on two tests on consecutive days (CA), and the decay slope of exploratory activity (HAB). The results have shown that under these conditions, PB did not produce adverse delayed cognitive effects, other than a delayed decrease in habituation of exploratory activity in the open field. A similar effect was observed with sarin and the opposite effect for the combination of sarin with PB. No effect of any of the treatments could be found in the conditioned or passive avoidance tests. Thus, this study does not support the hypothesis that delayed cognitive impairments experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level nerve agent exposure.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract Order DAMD17-00 200015.

Introduction.

Carbamate cholinesterase inhibitors provide additional protection, when used as pretreatment, from exposure to soman and tabun than that afforded by atropine and oxime alone (Dirnhuber et al., 1979) (Leadbeater et al., 1985) (Koplovitz et al., 1992) (Kluwe et al., 1987). On the basis of these findings, the quaternary cholinesterase inhibitor pyridostigmine bromide (PB) was adopted by USA and NATO armies as wartime pretreatment adjunct for nerve agent exposure. The therapeutic target for this application of pyridostigmine has been to maintain inhibition of plasma butyryl-cholinesterase (BuChE) between 20% to 40%. Large scale use of this premedication occurred during the Persian Gulf War, with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). This pretreatment and the possible exposure to low level sarin have been proposed by some to contribute to a conglomerate of symptoms experienced by Persian Gulf War veterans. The present study was designed to determine whether sub-symptomatic exposure to PB or low-dose sarin, alone or in combination, could elicit cognitive changes detectable 2 to 16 weeks after exposure to the agent.

Methods

Adult male Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment) and PB (the dose producing 20-30% inhibition of plasma BuChE, the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War). Experiments were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC-I.

Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates.

Animals were treated for three weeks with (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 x LD50 three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c.. There were 36 animals in each group, with three subgroups of 12 in each treatment that were studied 2, 4 or 16 weeks after treatment.

After tests for passive and active avoidance conditioning and open field activity were completed, rats were euthanized and the brain regions of interest were microdissected from frozen brain slices. These regions were homogenized, and aliquots were used for determination of tissue AChE activity (Ellman et al., 1961), ChAT activity (Fonnum, 1975), and quinuclidinyl benzilate (QNB) binding with saturation assays (Yamamura and Snyder, 1974).

Inhibited (passive) avoidance response: This response was measured in a "step through" apparatus (McGaugh, 1972), consisting of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel, and (c) a shock delivery unit adjustable for the intensity and duration of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hrs. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment leads immediately to the closing of a door and administration of footshock. Retention was tested after a 48-hr delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as "retention," a measure of memory of the single training session. The retention trials were set at a limit of 10 min. The times for animals not entering during the 10 min were recorded as 600 sec.

Conditioned avoidance response: A discrete trial, one-way conditioned avoidance response was observed using the apparatus and general procedure described by Russell and Macri (Russell and Macri, 1979). Two responses were studied: an innate escape response and a learned avoidance response. There was a maximum of 30 trials per session, with two sessions 24 hrs apart. The number of animals reaching criterion (6 consecutive avoidance responses) and the average escape and avoidance times per animal in both sessions were recorded for all experimental groups.

Open field locomotor activity: Activity was measured during a 20-min session in circular open field chambers of 60 cm diameter under low level red light illumination. This was done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. Each animal's movements were recorded with a video tracking and motion analysis system, consisting of a Sony CCD video camera (sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory (Figs 1,2). Distance traveled was summated at 1-min intervals, and these values were fitted by non-linear regression, using the Marquardt algorithm, to the model:

$$Y = A \cdot e^{-Bt} \quad (1)$$

where Y = distance moved (cm) and t = time after initiation of test (min). The values of parameters A (initial velocity, cm min⁻¹) and B (habituation, min⁻¹) were obtained as described above for every animal (Fig.3). Analysis of variance (ANOVA) was then performed for the two parameters using factors treatment (control, PB, sarin and sarin+PB) and time after treatment (2, 4, and 16 weeks). In addition, total distance traveled and mean distance to the arena's border (the wall of the chamber) during the entire test were also calculated for every animal.

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors (SE). Differences between group means were tested by ANOVA (general linear model) followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

Results

Dose Determination Studies: The LD50 of sarin was determined to be 125 µg/kg, sc. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/L had inhibition of plasma BuChE slightly greater than 20% on average, and was within the target effect set for these experiments (20 to 30% inhibition). The next higher PB concentration in drinking water (160 mg/L) induced a larger plasma BuChE inhibition (between 27 and 40 %). Thus the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No sign of toxicity, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination), was found in animals drinking water containing PB during the three-week treatment periods. The dose finding for sarin and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects, as described above, when given alone or in combination with PB (80 mg/L) in drinking water.

Body mass: Means of body mass, recorded daily on weekdays during the three weeks of treatment and the post-treatment weeks showed the expected increase with age, but no statistically significant differences were found among treatments.

Blood ChE activity: Measurements of red blood cells (RBC) AChE during the 3 drug treatment weeks, the pre-treatment week (two measurements) and 3 post-treatment weeks are shown in Fig 1. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the three weeks of treatment. Sarin and sarin plus pyridostigmine produced an average decrease in RBC AChE to 35% and 27% of pre-treatment, respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Open field locomotor activity:

Parameter A (initial velocity): No statistically significant difference was found among treatments at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for sarin plus PB (360.6 ± 19.9 cm min⁻¹) was significantly higher than the PB (272.8 ± 19.9 cm min⁻¹) and sarin (275.3 ± 20.8 cm min⁻¹) groups but not different from controls (309.5 ± 20.8 cm min⁻¹) (Fig 5).

Parameter B (habituation): No statistically significant difference was found among treatments at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter

means for sarin ($0.035 \pm 0.0088 \text{ min}^{-1}$) and PB ($0.046 \pm 0.0084 \text{ min}^{-1}$) were lower than for controls ($0.072 \pm 0.0093 \text{ min}^{-1}$), while sarin+PB ($0.101 \pm 0.0084 \text{ min}^{-1}$) was significantly higher than for all other groups (Fig. 4).

Passive avoidance: No difference between experimental groups was found in the time to enter the dark compartment 24 hrs after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response.

Conditioned avoidance: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test and the same parameters for animals that gained or lost criterion in the second day with regard to the first are shown in Figs 5 and 6. No significant difference was detected among experimental groups for the pooled data shown in the Fig.5, nor for any of the time points after treatment.

Brain regional AChE and ChAT activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon, 2 weeks after exposure to sarin.

Discussion

This study was designed to mimic the conditions of soldiers in the battlefield taking PB as a prophylactic treatment against nerve agent intoxication, with or without exposure to subsymptomatic levels of these agents. PB was administered in the drinking water to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans.

The lack of changes in the passive and conditioned avoidance paradigms under the conditions of this experimental model indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. On the other hand, habituation in the open field test, considered a primitive form of learning, was impaired for the PB and sarin groups at 16 weeks after treatment. This phenomenon was enhanced, however, in the group in which sarin treatment was combined with PB at the same time point. Given the present evidence, these phenomena are difficult to interpret and may require exploration of longer time points after treatment to define the possible interaction between sarin and PB on this particular type of behavior. Possible cognitive effects of the three treatments will be tested at later stages of this project by another learning test involving spatial orientation, the Morris water maze.

Learning impairments have been previously described in rats receiving PB (Liu, 1992) (Shih et al., 1991). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (11 mg/kg/day) or taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day) (Keeler, Hurst, and Dunn, 1991). Moreover, in these two earlier studies behavioral tests were performed within minutes of dosing, with no long-term follow up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of symptomatology, but assessment was limited to the period immediately following treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, the data obtained in this study on avoidance learning paradigms does not support the hypothesis that delayed cognitive impairments experienced by Persian Gulf War veterans could be due to PB, either alone or in association with low-level nerve agent exposure. The issue of possible effects of sarin or PB, and their interaction on the primitive form of learning represented by habituation on the open field test, deserves further exploration with other tests of spatial orientation.

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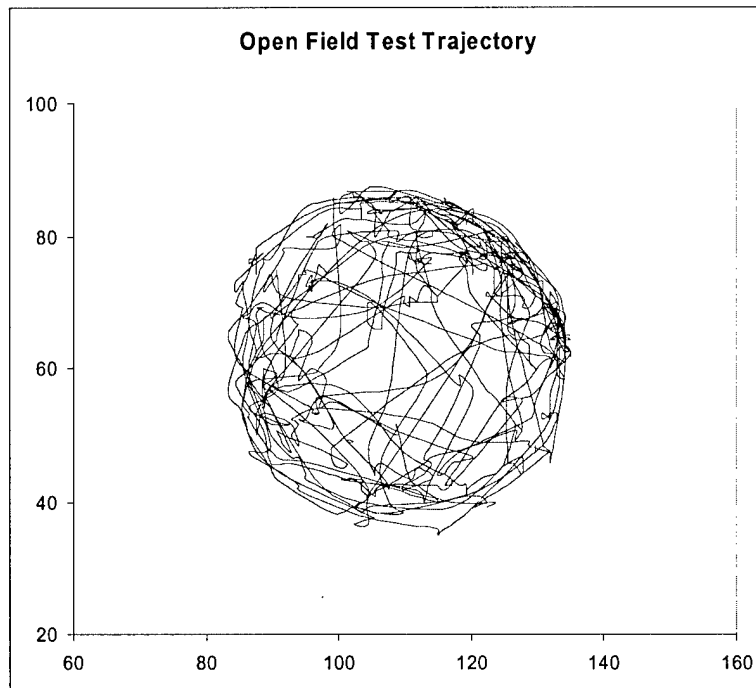


Figure 1: Open field test: trajectory of one animal over the circular arena as tracked by the video-monitoring system.

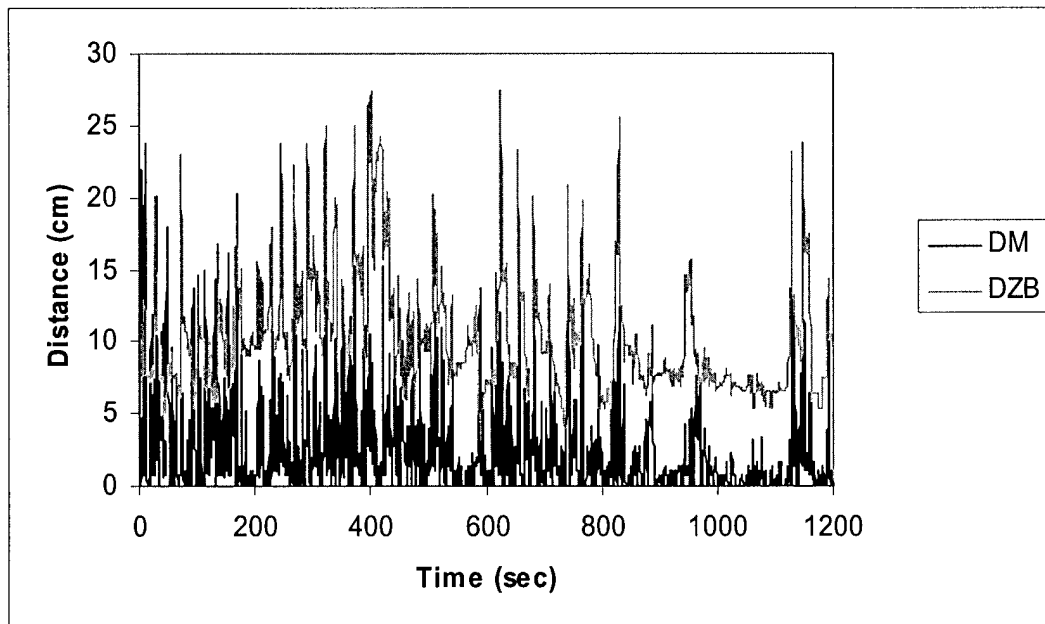


Figure 2: Open field test: distance moved (DM) and distance to arena's border (DZB) were computed every second throughout the test for the experiment shown in Fig. 1

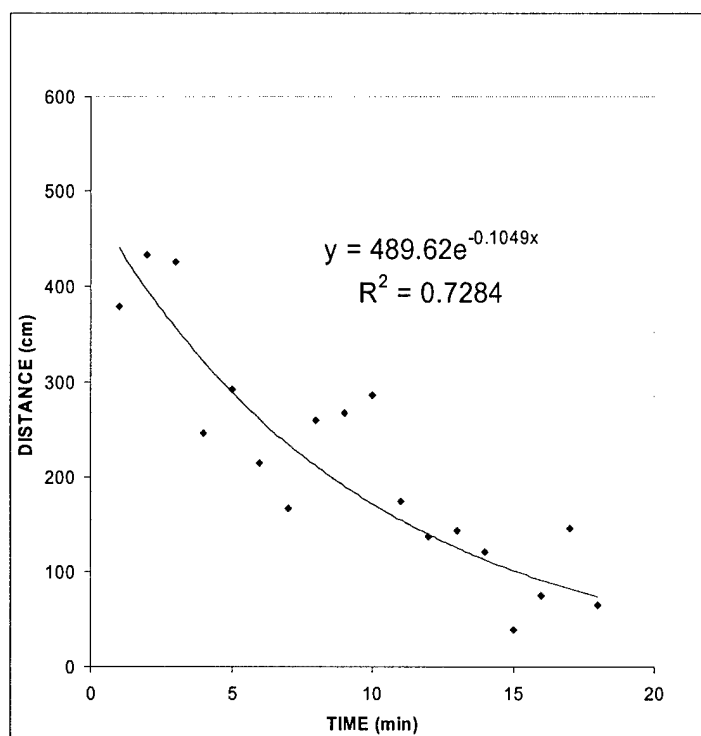


Figure 3: Parameters of monoexponential fits of distance moved over time in an open field (shown here for a single animal) were obtained in every case and calculated as described in Methods.

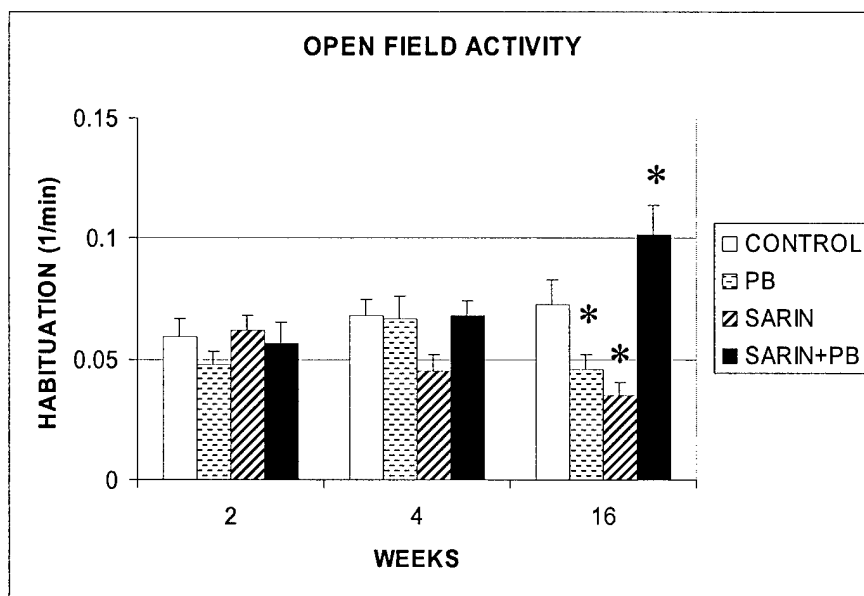


Figure 4: Means and SE of parameter B (habituation, rate constant). At 16 weeks following exposure, sarin and PB were lower than controls ($P < 0.01$ and 0.05 respectively), while sarin+PB was significantly higher than all other groups ($P < 0.001$ vs. sarin and PB, and $P < 0.025$ vs. controls). Parameter A (initial velocity, Y intercept) showed no differences among groups.

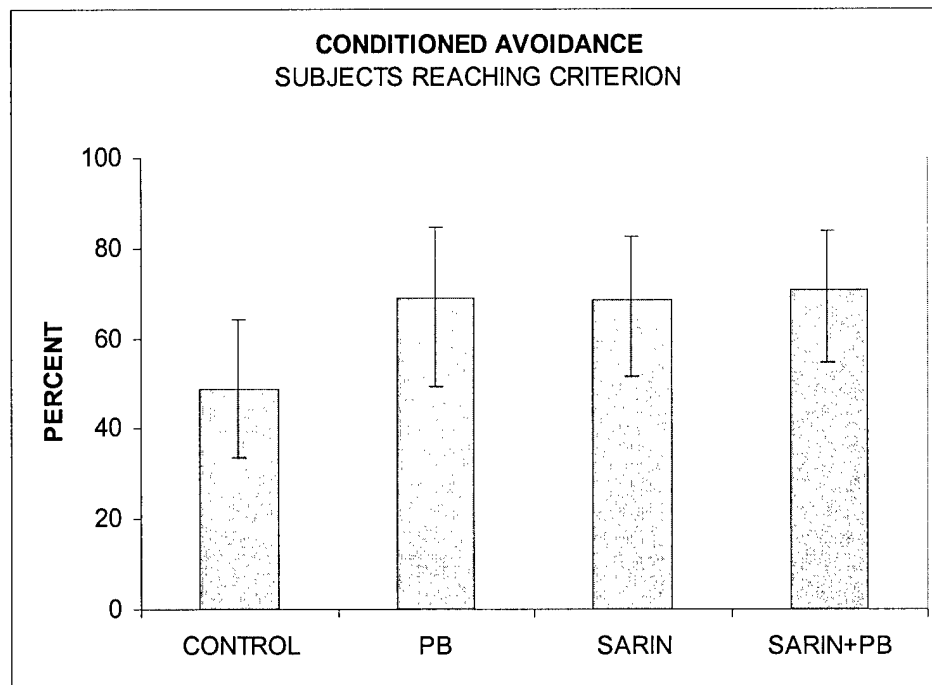


Figure 5: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test. There were no statistically significant differences between groups (pooled data from all times after treatment).

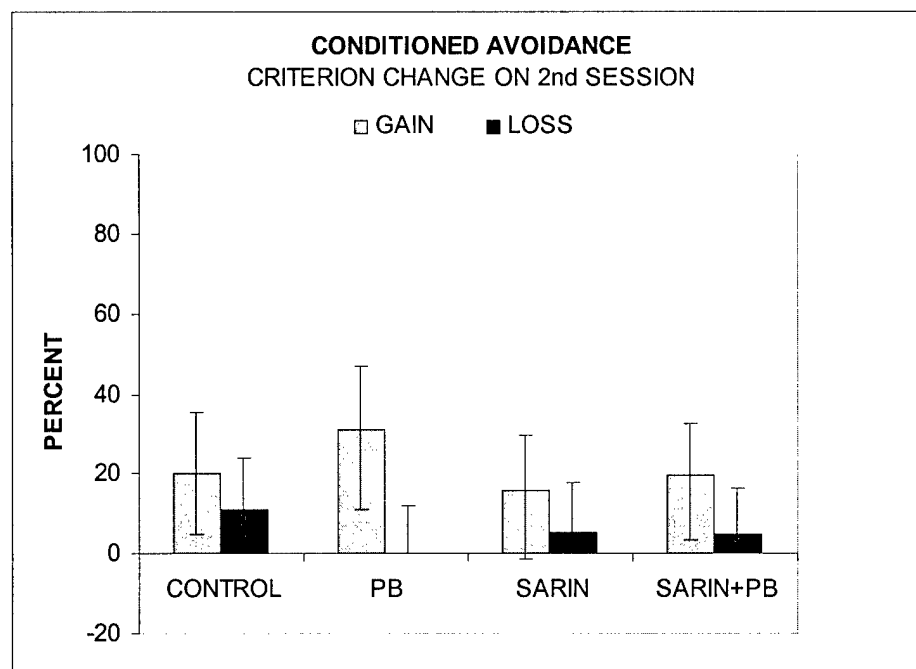


Figure 6: Percent and 95% confidence intervals of animals that gained or lost criterion in the second day when compared with the first. Pooled data from 2, 4, and 16 weeks after exposure.

PYRIDOSTIGMINE BROMIDE PREVENTS DELAYED NEUROLOGICAL EFFECTS OF LOW DOSE SARIN.

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Abstract

Many veterans of the Persian Gulf War complain of neurological symptoms, including balance disturbances, vertigo, and muscle aches and weaknesses, which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB) and/or sarin. The hypothesis that these agents, alone or in combination, elicit delayed neurological dysfunction was tested in Sprague-Dawley rats (CrI:CD(SD)IGSBR). Acoustic startle, locomotor activity in an open field, nociceptive threshold, and neural cardiovascular regulation were studied 2, 4, and 16 weeks after exposure to sub-toxic doses of PB and sarin, alone or in combination. Brain regional acetylcholinesterase (AChE) and cholinacetyltransferase (ChAT) activities and muscarinic receptor binding were studied in 10 critical brain regions. Two weeks after sarin, acoustic startle was enhanced, while distance explored in the open field decreased. These effects were absent with PB plus sarin or PB by itself. No effect on any variable was found at 4 weeks, while at 16 weeks an elevation of nociceptive threshold was found with the combination of sarin+PB. Mean arterial blood pressure, heart rate and gain of the baroreceptor reflex were similar across treatments. Brain regional AChE and ChAT activities were not affected at any time after any treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon at 2 weeks. In conclusion, PB protected against neurologic dysfunction in animals exposed to low dose sarin.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract Order DAMD17-00 200015.

Introduction

Exposure to pyridostigmine bromide (PB) and/or sarin has been implicated by some authors in the causation of a complex conglomerate of symptoms suffered by veterans of the Persian Gulf War (Haley, 2001). Exposure to PB resulted from its use as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979; Leadbeater et al., 1985; Koplovitz et al., 1992; Kluwe et al., 1987; Keeler et al., 1991). Large scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler, Hurst, and Dunn, 1991). Possible exposure to sarin may have occurred following explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001).

This study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf war veterans, could elicit delayed and persistent neurological dysfunction in experimental animals. An open field activity test was used to assess motor activity. Auditory startle and nociceptive threshold were assessed to determine the existence of possible dysfunction of the somatic nervous system since they have been shown to be affected by acute cholinesterase inhibition (Philippens et al., 1997; Russell et al., 1986). The baroreceptor mechanism of arterial blood pressure control was tested as an indicator of autonomic nervous system function because it includes both peripheral and central cholinergic steps in its circuitry (Brezanoff and Giuliano, 1982; Higgins et al., 1973). In addition, we analyzed, in relevant brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation respectively, as well as the expression of muscarinic cholinergic receptors. These assays were performed in the same animals that were subjected to the tests mentioned above.

Methods

Animals: Adult male Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment) and PB (the dose producing 20-30% inhibition of plasma BuChE, the degree of butyrylcholinesterase (BuChE) inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War).

Experiments were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC. Animals were treated during three weeks with (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 x LD50 three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c.

Open field locomotor activity: This was measured during a 20-min session in circular open field chambers of 60 cm diameter under low level red light illumination. This was done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a CCD video camera (Sony, Inc.), sensitive to the wavelength of light used, Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Total distance traveled and mean distance to the arena's border (the wall of the chamber) during the entire test were calculated for every animal.

Reactivity (startle response): Reactivity is defined as a response to a sudden brief and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been previously described (Silverman et al., 1988; Russell and Macri, 1979). In this procedure the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom designed computer program delivers a controlled sound and integrates and digitizes the movement-related electrical signal. Quantification of the response is provided in arbitrary force units. In the currently reported experiments, 20 trials were performed at fixed intervals of 10 seconds.

Nociceptive threshold: The procedure to measure nociceptive threshold used in these experiments has been previously described (Crocker and Russell, 1984) and utilizes reaction to a mild electric foot shock as its measure. It involves the "up and down" method described by Dixon (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 sec at 10-sec intervals. The shock intensities were available in a range from 0.05 mA to 4.0 mA and arranged in a \log_{10} scale at 0.1 \log_{10} units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animals response on each trial. A "flinch" was defined as an elevation of 1 or 2 paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

Cardiovascular regulation: Animals were instrumented with arterial and venous femoral indwelling catheters under halothane anesthesia for recording of arterial blood pressure and infusion of drugs respectively. They were then allowed to recover from anesthesia in a Bollman cage, where they remained conscious but restrained during the rest of the test. Arterial blood pressure (BP) was transiently altered by pulse injection of phenylephrine (5 to 10 $\mu\text{g/kg}$, i.v.) and sodium nitroprusside (20 to 50 $\mu\text{g/kg}$, i.v.). Heart rate (HR) was continuously recorded along with arterial blood pressure, and regressions of HR on BP were calculated from data obtained before and after the pulse injections of phenylephrine and nitroprusside, as an estimate of the baroreceptor gain.

Neurochemistry: Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates. After the tests described above were completed, rats were euthanized, and the following brain tissue regions were microdissected from frozen brain slices: somato-sensory, temporal, and pyriform cortex, hippocampus, caudate-putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These regions were homogenized, and aliquots used for determination of tissue AChE activity (Ellman et al., 1961), ChAT activity (Fonnum, 1975), and quinuclidinyl benzilate (QNB) binding with saturation assays (Yamamura et al., 1974).

Experimental groups: Animals were divided into 4 groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline. Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. Group 3 animals received tap water and were injected with sarin (62.5 $\mu\text{g/kg}$, sc, equivalent to 0.5 LD₅₀). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above. PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 hour. At 09:00 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 $\mu\text{g/kg}$, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 17:00 hours on Friday of the third week. There were 36 animals in each group, with three subgroups of 12 in each treatment group that were studied 2, 4 or 16 weeks after treatment.

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

Results

Immediate treatment effects.

The dose finding for sarin, and the combination of sarin and PB indicated that 0.5 LD₅₀ sarin was the highest dose devoid of acute toxic effects, as described above, when given alone or in combination with PB (80 mg/L in drinking water). Means of body mass, recorded daily during weekdays, through the three weeks of treatment showed the expected increase with age, but no statistically significant differences were found among treatments.

PB induced a pronounced decrease in RBC AChE activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the

three weeks of treatment. Sarin, and sarin plus PB produced an average decrease in RBC AChE to 35% and 27% of pre-treatment respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Delayed treatment effects.

Motor performance in the open field test: ANOVA was significant at 2 weeks after treatment for total distance moved within the arena. Multiple contrasts indicated that the sarin group mean was significantly lower than controls (Fig. 1). No difference vs. controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

ANOVA was also significant ($P < 0.05$) at 2 weeks after treatment for the average distance to the arena's border. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and sarin+PB (9.05 ± 0.45 cm), but not different from controls (8.63 ± 0.64 cm).

Nociceptive threshold: No statistically significant difference among groups was found for the flinch response to the test at 2 and 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher LSD test, $P < 0.05$) showed that the nociceptive threshold of the animals that received the combination of sarin and PB was significantly higher than all other groups. ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group than in the PB, and sarin+PB groups, but not significantly different from controls. At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the sarin+PB group had a significantly higher threshold than all other groups. Data are presented in Fig. 2 for the jump response.

Reactivity (acoustic startle): A significant increase of sarin-treated animals against the controls in the average motor response over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (Fig. 3). In this case, the mean of the sarin group was significantly higher than all others. No difference among group means was present at 4 or 16 weeks after treatment.

Cardiovascular regulation: Typical responses of BP and HR to phenylephrine and nitroprusside are shown in Fig 4. The highest phenylephrine dose elicited atrioventricular blockade (Fig 4, top) followed by nodal, and in some cases ventricular ectopic rhythms. The coefficient of the regression of HR on BP calculated from hypertension data prior to the A-V block yielded values similar to that of the regression obtained from hypotensive episodes. For that reason both sets of data were pooled in one analysis (Fig 5). In another analysis, only data from hypertensive episodes (including the A-V block) was used. None of the differences between experimental groups reached statistical significance.

Brain regional AChE and ChAT activities and QNB binding: Enzymatic activities were not affected at any time after any treatment, but QNB binding was reduced in hippocampus, caudate-putamen and mesencephalon, 2 weeks after exposure to sarin (data not shown). However, no changes were detected 4 and 16 weeks after treatments.

Discussion

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test two weeks after discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Russell, Booth, Lauret, Smith, and Jenden, 1986; Overstreet, 1977). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease particularly pronounced in caudate-putamen, hippocampus and mesencephalon. Downregulation of muscarinic receptors may have played a role in the behavioral phenomena described above since this was their only neurochemical correlate.

Both the depressed locomotor activity and enhanced startle response induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from sarin

lethality (Harris and Stitcher, 1984). Contrary to previous reports (Servatius et al., 1998), PB did not elicit delayed changes in acoustic startle.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990; Russell, Booth, Lauretz, Smith, and Jenden, 1986), and the reverse is true of hypercholinergic states (Shih and Romano, 1988). The facts that both the flinch and the jump response were enhanced only 16 weeks after treatment is difficult to interpret since neither cholinesterase activity nor cholinergic receptor binding were found altered at this time. Secondary delayed effects of the initial exposure to this drug combination may be at work and deserve further exploration.

The lack of changes in baseline levels of arterial blood pressure and heart rate as well as in the gain of the baroreceptor response are indications that the central and peripheral cholinergic steps involved in cardiovascular regulation were intact in the experimental groups under study.

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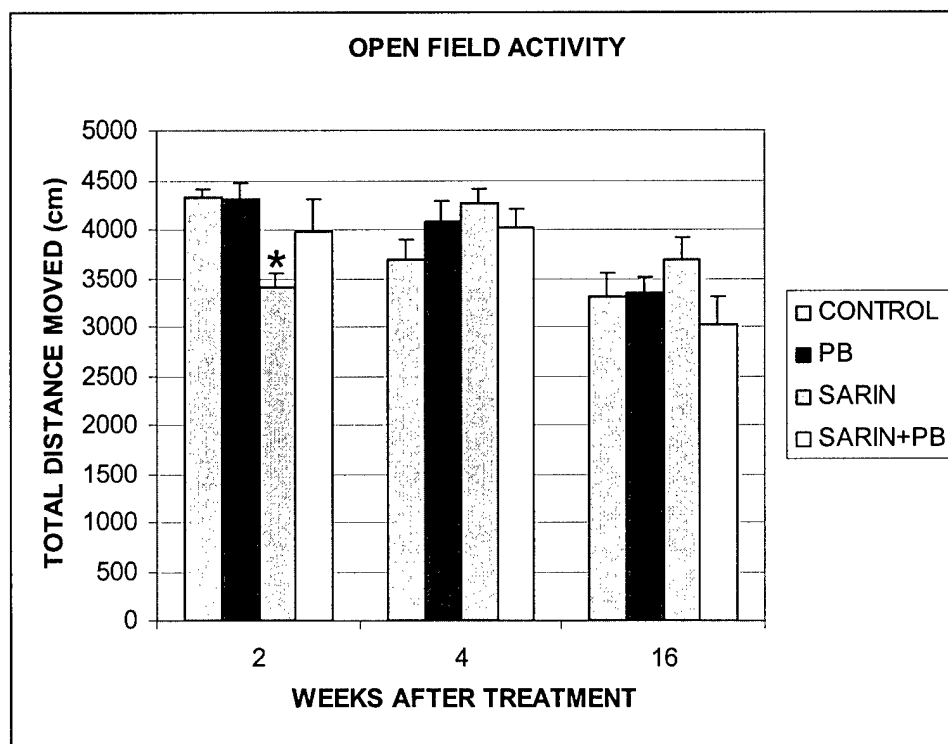


Figure 1: Means and SE of total distance moved in the open field for all experimental groups (12 rats per group). The sarin mean was significantly lower than controls at 2 weeks ($P < 0.05$, ANOVA and Fisher's multiple comparisons LSD test).

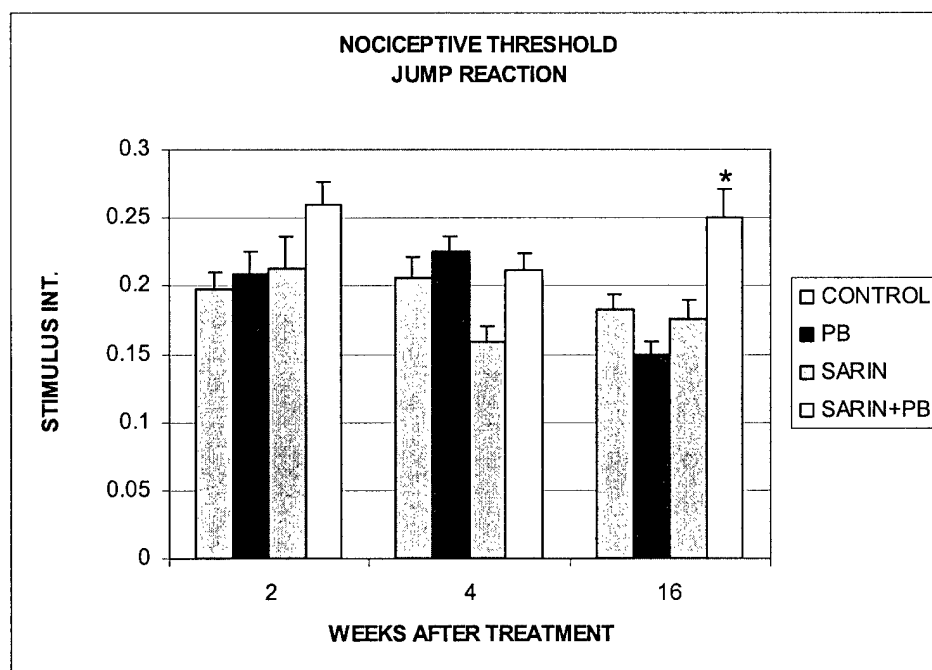


Figure 2: Means and SE of jump nociceptive threshold for all experimental groups (12 rats per group). The sarin+PB mean was significantly higher ($P < 0.05$, ANOVA and Fisher's multiple comparisons LSD test) than all others at 16 weeks post-treatment.

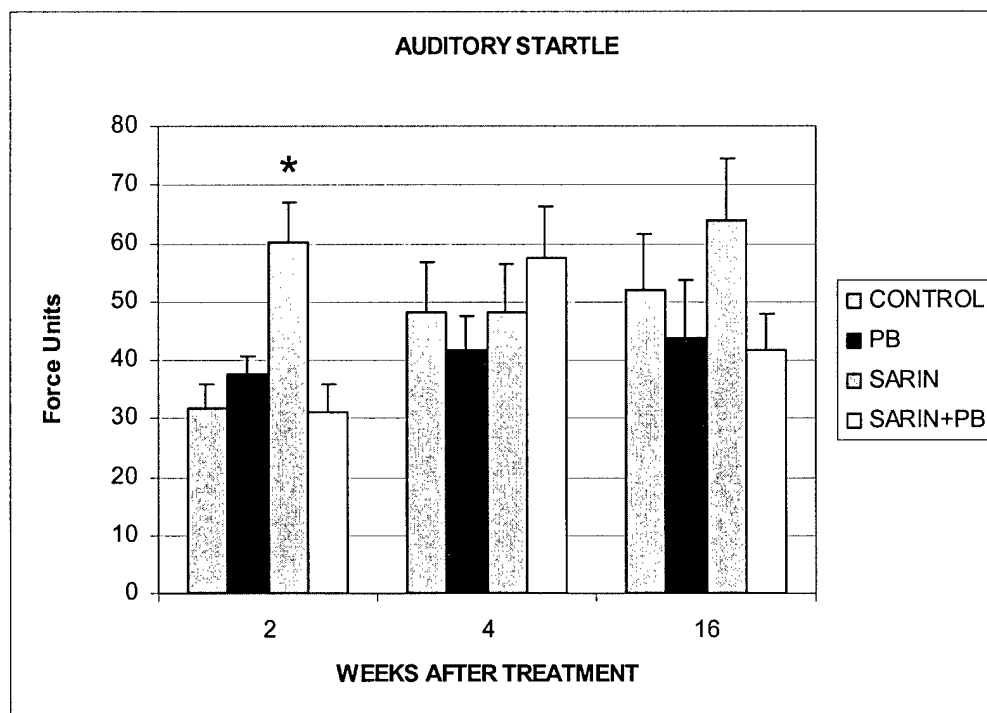


Figure 3: Means and SE of maximal response to acoustic startle for all experimental groups (12 rats per group). The sarin mean was higher than controls at 2 weeks after treatment ($P < 0.005$, ANOVA and Fisher's multiple comparisons LSD test).

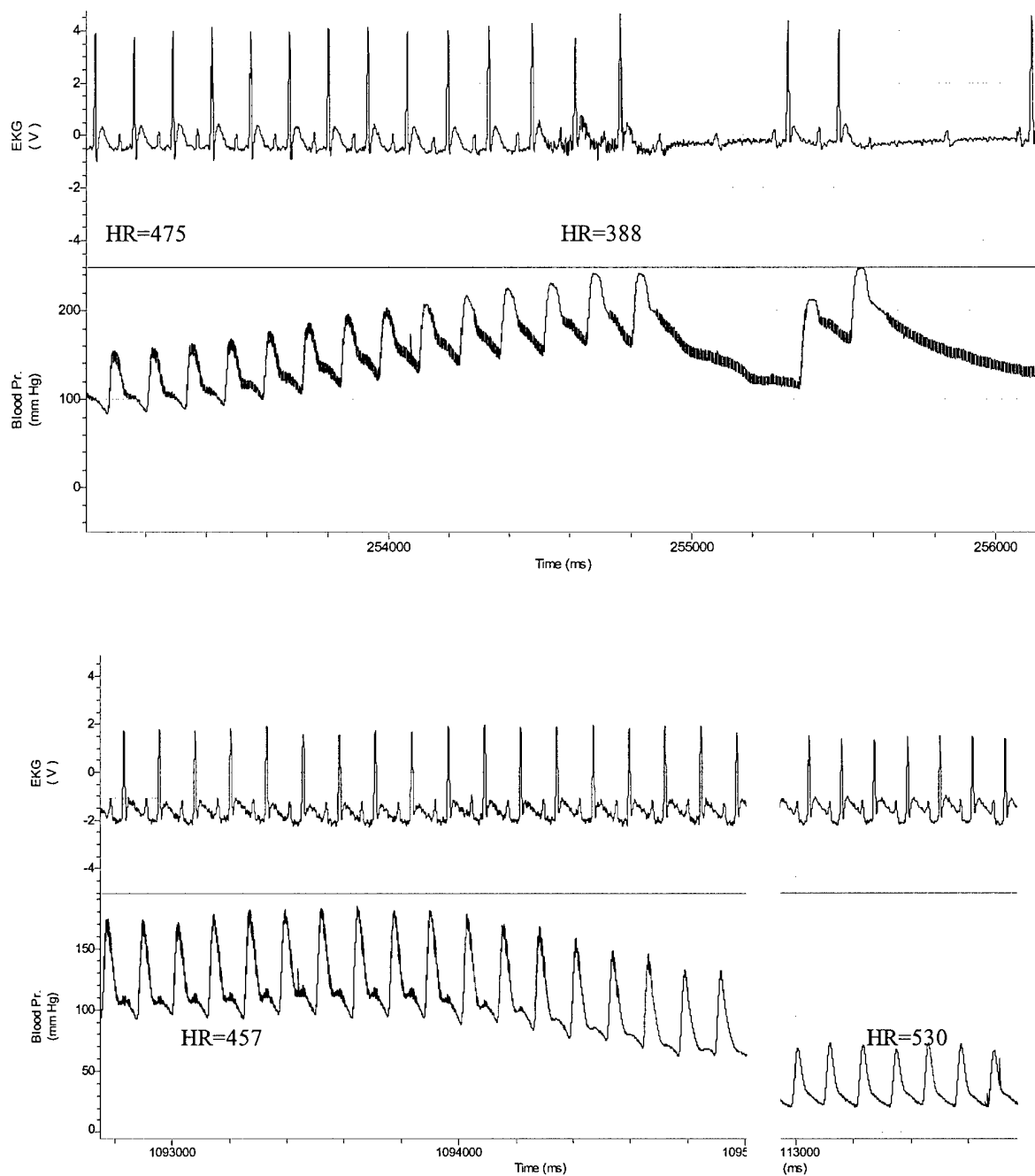


Figure 4: Representative baroreceptor mediated heart rate responses to pharmacologically induced hyper- or hypotension. TOP: Progressive hypertension and sinus bradycardia after phenylephrine (PE), followed by A-V block and nodal bigeminal rhythm. BOTTOM: Progressive hypotension and tachycardia following nitroprusside (NP). Two doses of each drug were given to every animal and the regression of HR on MABP calculated with or without inclusion of beats beyond the A-V block.

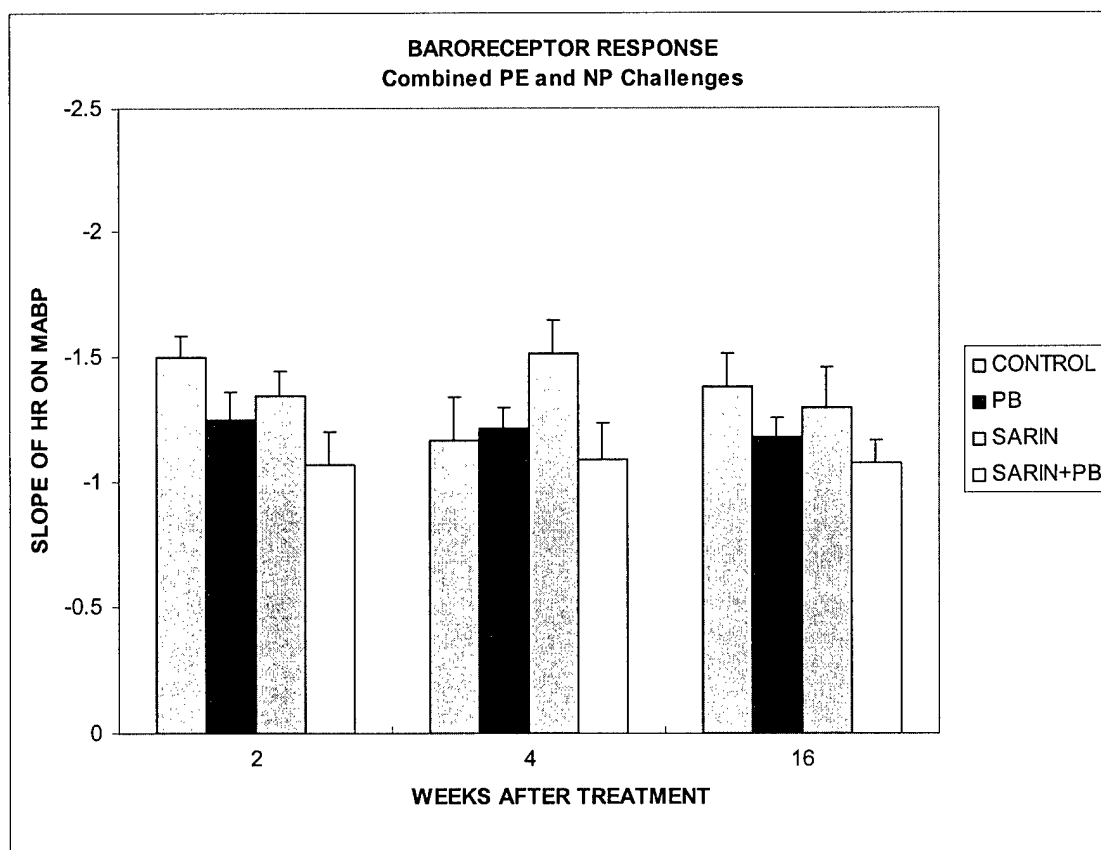


Figure 5: Mean and SE of slopes of the linear regression of HR on MABP for all PE and NP challenges excluding heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.